

**THE INFLUENCE OF PLOIDY ON THE PRE- AND POSTIMPLANTATION  
DEVELOPMENT OF MOUSE EMBRYOS**

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## **DECLARATION**

Except where noted, I declare that the work presented within this thesis is my own.

**CAROLINE CECILIA HENERY**

## ABSTRACT

All human polyploid conceptuses develop abnormally during their gestation and the majority are aborted in the first trimester. A few embryos, however, can reach term but they die soon after birth. The importance of this condition in human embryonic loss and its clinical significance in the development of gestational trophoblastic disease merits an investigation into the aetiology of polyploid abnormal development and death.

Since the number of pre- and postimplantation human polyploid embryos available for analysis is very small and relatively few are available at any one centre, I have used appropriate experimental mouse models to facilitate a large scale investigation. One aim was to analyse the influence of ploidy on the cleavage rate of preimplantation embryos. A deviation from the optimum embryonic (diploid fertilized) cell number during this time might explain the poor development and failure of genetically abnormal embryos. Therefore, experimentally produced haploid, diploid, triploid and tetraploid embryos were analysed together with appropriate control embryos. In addition, the analysis of parthenogenetic embryos and diandric and digynic triploid and homozygous tetraploid embryos during this time would also enable the influence of the parental genomes on development to be investigated. I also wished to examine whether a predictable relationship existed between ploidy and cell size and number in the tissues of postimplantation mammalian polyploid embryos. Confirmation of such a relationship may explain the abnormal morphological features encountered in some of these embryos and the premature death of all triploid and tetraploid mouse embryos, and the majority of human embryos with similar genetic abnormalities.

My results show that the duplication of one or both parental genomes leading to the triploid or tetraploid status, respectively, still allows apparently normal preimplantation development to occur. However, the loss of a haploid genome from the diploid status is invariably detrimental to normal development. My results confirm that the presence of a maternal genome is important for normal early embryogenesis since parthenogenetic diploid embryos and diandric

and digynic triploid embryos developed as well as fertilized diploid embryos during the pre- and very early postimplantation period. No genomic imprinting differences were observed between diandric and digynic triploid embryos during the preimplantation period. A predictable relationship was, however, established between cell and nuclear volume and ploidy in postimplantation triploid and tetraploid embryos. In tetraploids at least, the increase in cell size observed in relation to ploidy was also related to a reduced cell number in their tissues, so that tetraploid body size was closer to the normal rather than of gigantic proportions. However, their tissue morphology remained reasonably normal in appearance.

These results lead me to conclude that the abnormal development of polyploid mouse embryos is not related to an abnormal preimplantation development. The abnormalities encountered in polyploid embryos may be the consequence of an increase in cell size related to a decrease in cell number brought about by regulatory factors in an attempt by the embryo to maintain a normal body size. However, this is unlikely to lead to their death since in tetraploids at least, where the most advanced stages of postimplantation development are achieved, none of the abnormalities encountered appeared to be life threatening. By contrast, in the case of triploids, the geometrical problems associated with triploidy are probably not as important to their abnormal development and death as the fact that they are genetically unbalanced. Strain and species differences are also likely to complicate our understanding of the pathology of polyploidy.

Finally, in order to obtain a more efficient protocol than presently available to investigate the influence of ploidy on development, methodological consideration has been given to the production of parthenogenetic embryos by electrical pulse stimulation. In the light of previous parthenogenetic studies, I aimed to investigate the cytogenetic response of mouse eggs to this stimulus in order to establish whether it was a reliable means of producing euploid parthenogenetic embryos. My results show that there was no increase in the incidence of aneuploidy with this type of stimulus and that this stimulus should be considered as a valuable means of producing euploid parthenogenetic embryos.



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## CHAPTER 1

### INTRODUCTION



### 1.1 INCIDENCE OF CHROMOSOMAL ABERRATIONS IN MAN

Reproduction is fundamental to every species, however, in man this process is fraught with many problems. It has been estimated that between 10-50% of all human conceptions are chromosomally abnormal (Bond and Chandley, 1983). The reason for such disparity in estimates is the absence of any direct analysis at this stage. To estimate accurately the incidence of genetic abnormalities at conception, requires the analysis of the gametes themselves. In animals, where this is possible, it is known that the incidence of chromosomal errors is low. Techniques have been developed, however, to overcome the difficulties encountered when dealing with human material. Fertilization of hamster eggs *in vitro* with human spermatozoa, for example, enables human sperm chromatin to be visualised (Rudak et al., 1978; Martin et al., 1982), and it has been found that approximately 8% of human sperm may contain chromosomal aberrations. Recent advances in *in vitro* fertilization techniques have made available "spare" oocytes and preimplantation embryos which can be analysed for chromosomal abnormalities. Such studies have found a high proportion (sometimes one-third) of embryos cytogenetically abnormal (Angell et al., 1988). These techniques may allow a better estimate of the chromosomal abnormalities at conception in man.

It has been estimated that approximately 15% - 20% of all clinically recognised pregnancies end in spontaneous abortion (Roth, 1963; Warburton and Fraser, 1964; Inhorn, 1967), with the majority of these occurring in the first trimester (Boué and Boué, 1973). The loss is higher, however, when spontaneously aborted, clinically unrecognised pregnancies are taken into account (Carr, 1971a, b). A better estimate of human conceptional loss may result from the detection of early pregnancies before a proportion of them are aborted. Early diagnosis can be carried out using ultrasound, chorion villus sampling, amniocentesis, and maternal biochemical screening techniques (Neilson and Gosden, 1991). Maternal biochemical screening techniques, for example, can be carried out using immunoradiometric assays specific for detecting human chorionic gonadotrophin (hCG) just after implantation. In the past, these assays have only been sensitive to large increases in the B subunit of hCG. Surprisingly, the estimates of early abortions have been as high as 60% of all conceptions

(Edmonds et al., 1982). Using an assay highly sensitive to BhCG, Wilcox et al.(1988) found that 22% of (221) women in their study experienced a spontaneous abortion of a clinically unrecognised pregnancy. Together with clinically recognised spontaneous abortions, the incidence of conceptional loss in this group of women amounted to 31%. This figure would have been higher if preimplantation losses could have been detected.

Indications of the underlying causes responsible for the high incidence of human pregnancy wastage have come from the cytogenetic analysis of recognised spontaneous abortions. It has been established that about half of these spontaneous abortions are chromosomally abnormal (Boué et al, 1975; Lauritsen, 1976; Hassold et al., 1980; Kajii et al., 1980; Warburton et al., 1980; Hassold and Chiu, 1985; Uchida and Freeman, 1985), while the rest are genetically normal embryos. The latter may abort for a number of reasons, including poor hormonal control of the pregnancy (Blumenfeld and Ruach, 1992), and immunological hostility. These abortions are also worthy of analysis, but they are out of the scope of this thesis. Of the abortions that are chromosomally abnormal, the majority (60-70%) have genetically lost or gained one or more chromosomes and are, therefore, said to be aneuploid. About a quarter of the abortions are the result of having gained an extra one or more haploid sets of chromosomes and are said to be polyploid. The remaining 5-10% of abortuses with a chromosomal abnormality are comprised of trisomic and polyploid mosaics, translocations and chromosome breakages (see Figure 1.1).

## 1.2 ANEUPLOIDY AS A CAUSE OF SPONTANEOUS ABORTION

Aneuploidy in man accounts for approximately 70% of all chromosomal abnormalities and the majority of these (about 50%) are autosomal trisomies (Bond and Chandley, 1983). The underlying mechanism of aneuploidy is chromosome malsegregation, usually resulting from non-disjunction at:

- i) Maternal meiosis I
- ii) Maternal meiosis II

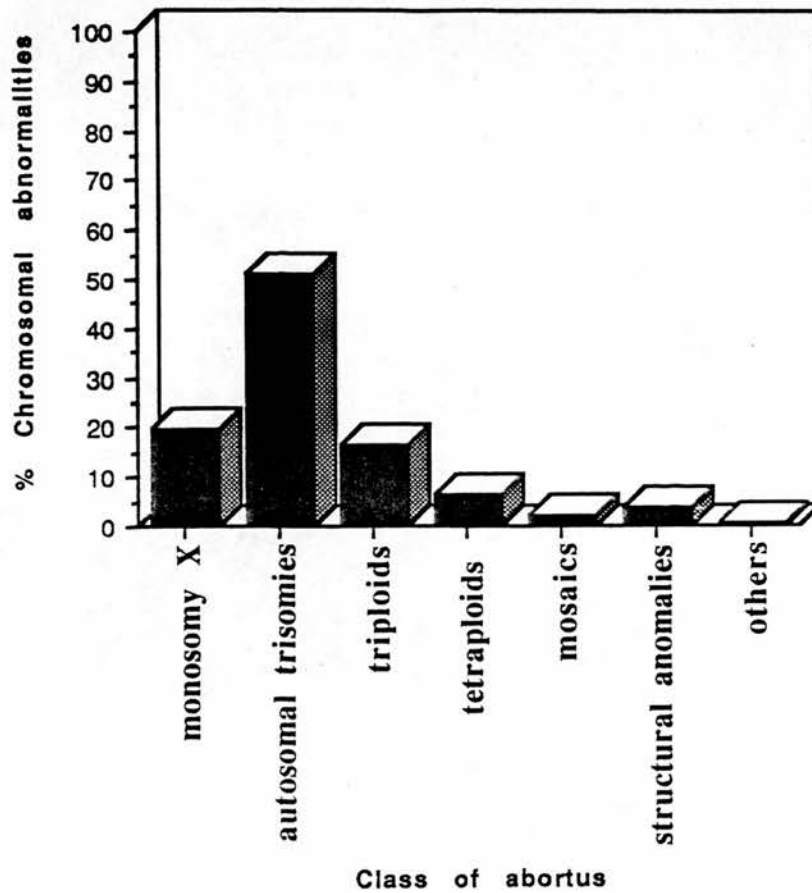


Figure 1.1

Frequency of different types of chromosomal abnormalities found in six spontaneous abortion surveys (assuming 50-60% of human abortuses are chromosomally abnormal): Lauritsen et al. (1972); Therkelsen et al. (1973); Kajii et al. (1973); Boué et al. (1975); Creasy et al. (1976); Hassold et al. (1980). Reviewed in Bond and Chandley (1983).

- iii) Paternal meiosis I
- iv) Paternal meiosis II

The single most common chromosomal abnormality in man is sex-chromosome monosomy 45 X, which accounts for about 20% of all spontaneous abortions but is occasionally compatible with live birth (Boué et al, 1975; Kajii et al., 1980; Meulenbroek and Geraedts, 1982; Lin et al., 1985). The most commonly encountered autosomal trisomy in humans is trisomy 16 which accounts for about a third of all autosomal trisomies (Bond and Chandley, 1983). If it is assumed that non-disjunction results in an equal probability of a monosomic or a trisomic conception, then autosomal monosomies are severely lacking in clinically recognised spontaneous abortions. This indicates that they are lethal at a much earlier stage of development (Ohama and Kajii, 1972), similar to a situation found in mice (Gropp et al., 1976). Mosaic trisomies occur as a result of an error in cleavage in pure trisomies, and the contribution that they make to the complement of chromosomal abnormalities in spontaneous abortions varies from 0-5% (Boué et al., 1985).

In contrast to man, the incidence of spontaneous aneuploidy at conception and in the early stages of development in animals has been well established as being about 1-2% (see Bond and Chandley, 1983; Dyban and Baranov, 1987). Overall, the incidence of chromosomal abnormalities in some animals may be just one-fifth of the level found in man (Bond and Chandley, 1983).

### 1.3 AETIOLOGY OF ANEUPLOIDY

The aetiology of such exceptionally high rates of aneuploidy in humans remains obscure. An increase in aneuploidy has been linked to errors arising in the human maternal germline associated with ageing. The majority (75-90%) of chromosomal errors occur at meiosis I with a smaller proportion occurring at meiosis II in the oocyte, while the contribution paternal errors and cleavage errors make is considerably less (Jacobs and Hassold, 1980; Hassold et al., 1984a). Penrose (1933) first established, through case studies on the incidence of trisomy 21,



that the incidence of aneuploidy increased with advanced maternal age. Further work in this area has established similar maternal age-related trisomies and, in particular, it has been found that there is an increase in the non-disjunction of smaller acrocentric chromosomes with advanced maternal age (Hassold et al., 1980; Hassold et al., 1984b). Conversely, however, an inverse relationship between the incidence of XO conceptuses and advanced maternal age apparently exists (Kajii and Ohama, 1979; Warburton et al., 1980; Hassold and Chiu, 1985). It has been suggested that in this case, some XO conceptions may be due to chromosome loss following anaphase lagging at meiosis or mitosis and not the result of non-disjunction. A paternal age-related relationship with aneuploidy has been more difficult to establish.

Various hypotheses have been put forward to account for the phenomenon of age-related aneuploidy. The suggestion that, in older mothers, there is a relaxation in the early expulsion of chromosomally abnormal embryos gains little attention now (Ayme and Lippman-Hand, 1982). In animals, it has been suggested that meiotic non-disjunction in the oocytes of older mothers may be due to inadequate tubulin polymerization, causing incomplete formation of the spindle microtubules (Sugawara and Mikamo, 1983). Evans (1967) suggested that nucleolus persistence in the oocytes of aged mothers interferes with normal chromosome pairing and predisposes the chromosomes to malsegregate. Henderson and Edwards (1968) proposed a "production-line" theory in which the increase in non-disjunction in eggs of aged CBA strain female mice was associated with a decrease in chiasma frequency in eggs which were selectively conserved until later life. This would lead to an increase in the number of univalents at meiosis I and result in a high rate of aneuploid conceptions in aged females. The findings on chiasma frequency and univalent incidence has been confirmed in that strain of mice by others (see, for example, Speed, 1977), but the association between univalent incidence at meiosis I and the frequency of non-disjunction at meiosis II has not been confirmed (Polani and Jagiello, 1976).

Maternal age-related aneuploidy exists in some strains of mice (Gosden, 1973; Martin et al., 1976; Maudlin and Fraser, 1978; Tease, 1982), but so far it appears not to be as common as it is in humans (Bond and Chandley, 1983). Brook et al. (1984) have specifically analysed CBA female mice as they have a short reproductive lifespan, show an age-related increase in aneuploidy and ovulate all of their eggs by the end of their reproductive life, which is similar to the situation in the human female. They have suggested that abnormal segregation of chromosomes is a phenomenon of changing oestrous cycle patterns, brought on by biological ageing rather than chronological ageing, and that such biological ageing could apply to women who undergo early menopause.

While there is clearly an age-related incidence of aneuploidy, the majority of spontaneous abortions occur in women of 34 years or less (Juberg, 1983), and most data on the incidence of aneuploidy associated with maternal age indicates that it follows a bimodal distribution (Boué et al., 1975). This is indicative of the presence of an age-independent mechanism of aneuploidy.

It has been proposed that ageing of the meiotic spindle in postovulatory aged eggs predisposes the chromosomes to malsegregate (Eichenlaub-Ritter et al., 1986; Webb et al., 1986). This would seem to confirm Rodman's (1971) observation that, in her study, chromatid disjunction occurred in 15% of aged eggs of mice compared to an incidence of 1.8% in recently ovulated eggs. However, Eichenlaub-Ritter and colleagues in their study never observed a chromosome independent of its spindle. Furthermore, experiments with parthenogenetic F1 (C57BL x CBA) mouse embryos have shown that the incidence of aneuploidy does not increase with postovulatory age (O'Neill and Kaufman, 1988). The influence that spermatozoa ageing has on the incidence of aneuploidy is also still unclear. Sapp and Martin-DeLeon (1992) found that the frequency of spermatozoa-derived hyperhaploidy in mouse embryos fertilized with aged spermatozoa differed significantly from that of unaged spermatozoa. This is in contrast to the results of Munne and Estop (1991), who found no effect on the incidence of aneuploidy when spermatozoa were aged *in vitro*.

Aneuploidy has also been found to be induced by a variety of physical and chemical agents. In the case of maternally-related aneuploidy, these may act throughout the lifespan of the eggs or only at the stage of egg maturation (for review, see Bond and Chandley, 1983; Hansmann, 1983). Kaufman (1985) suggests that exposure to spindle active agents may be important in the aetiology of aneuploidy where no other obvious cause is recognised. Examples where the influence of maternal ageing is the likely aetiological factor, or when conception occurs in a subject bearing a balanced chromosome translocation would not fall into this category. In this respect, it has been demonstrated that some anaesthetics may cause an increase in the incidence of chromosomal aberrations in mouse oocytes (Kaufman, 1977). Alcohol or its primary metabolite, acetaldehyde, shares the same physical and chemical properties as anaesthetics, and experimental studies have shown that an intragastric administration of a dilute solution of alcohol to female mice, at the time of the first or second meiotic division of the oocyte, results in a high number of aneuploid embryos (Kaufman, 1983; Kaufman and Bain, 1984; O'Neill and Kaufman, 1987a). High levels of aneuploidy are also seen when mouse oocytes are parthenogenetically activated with ethanol, and the incidence of aneuploidy is directly related to the duration of exposure to the ethanol (Kaufman, 1982; O'Neill and Kaufman, 1989). It has been hypothesised that this agent probably interferes with the cytoskeletal elements of the spindle apparatus or its precursor elements (Kaufman, 1985) and ultrastructural analysis appears to confirm this (O'Neill et al., 1989). While a wide variety of other chemical and physical stimuli are capable of inducing mammalian eggs to undergo parthenogenetic activation, the cytogenetic analysis of these embryos has shown that parthenogenesis *per se* is not associated with an increase in the incidence of aneuploidy (Kaufman, 1983). The fact that alcohol has been shown to be teratogenic to the developing fetus both in the human and in numerous experimental studies (Pratt, 1982), and is widely available to females (unlike a variety of other agents alledged to cause aneuploidy), give it grounds for serious consideration as a possible causative agent.

#### **1.4 POLYPLOIDY AS A CAUSE OF SPONTANEOUS ABORTION**

The other major group of chromosomal abnormalities found in spontaneous abortions are the polyploids. My thesis is particularly concerned with the development and morphology of this group of chromosomal abnormalities since the contribution they make to human wastage is a substantial one, and very few studies have been undertaken using this material.

Polyploidy accounts for about 25-30% of all chromosomal abnormalities in clinically recognised spontaneous abortions. Triploidy (the addition of a haploid set of chromosomes to the normal diploid genome) accounts for about 20% and is three times as common as tetraploidy (double the normal genome). In a relatively high proportion of cases, genetic techniques including chromosome banding, polymorphic enzyme loci analysis, human lymphocyte antigen specificities and restriction fragment length polymorphisms, have allowed the identification of the extra parental origin of the haploid and diploid sets of chromosomes in polyploid embryos to be established. Commonly, diandric triploid conceptuses are associated with hydatidiform degeneration of the placenta, and develop as "partial" hydatidiform moles. "Complete" moles, which have an exclusively diploid paternally-derived genome, have a greater predisposition for malignant transformation to form a choriocarcinoma than "partial" moles. In some women, benign ovarian teratomas occur and result from the parthenogenetic activation of ovarian oocytes (Linder et al., 1975). These teratomas, therefore, develop exclusively from a maternally-derived genome.

#### **1.5 THE INCIDENCE OF SPONTANEOUS POLYPLOIDY IN ANIMALS**

The incidence of polyploidy in animals is dependent on the species and strain but is generally very low (Austin, 1960; Baranov, 1976; Dyban and Baranov, 1987 for review). In laboratory rabbits and rats, for example, the incidence of triploidy in preimplantation embryos is less than one per cent (Dyban and Baranov, 1987). Most of the information that has accumulated on polyploidy in animals, however, has been on mice. For example, Beatty (1957), estimated the overall spontaneous incidence of triploidy in 3.5-day mouse embryos to be less than 1%. In one particular cross of mice, however, (silver strain female mice mated to non-silver strain males),



the incidence was found to be much higher at 5.7% (Beatty, 1957). A high incidence of triploid conceptuses is also seen in matings with LT/Sv strain female mice, since at 6 weeks of age, the females of this strain, ovulate about 50% of their eggs as primary oocytes (Kaufman and Howlett, 1986; Speirs and Kaufman, 1988) which are capable of being fertilized to give rise to digynic triploids (O'Neill and Kaufman, 1987b). Tetraploidy is also very rare in mice, for example, in 3671 analysable 3.5 day mouse embryos, only 5 were found to be, on cytogenetic analysis, tetraploid (Beatty and Fischberg, 1951, 1952).

## 1.6 POLYPLOIDY IN HUMANS

### 1.6.1 TRIPLOIDY

Triploidy occurs in about 1% of all recognised human conceptions and its most common mechanisms of induction are:

- i) the fertilization of a normal haploid oocyte by two haploid spermatozoa (diandric triploidy).
- ii) the fertilization of a normal haploid oocyte by an unreduced diploid spermatozoon (diandric triploidy).
- iii) the fertilization of an unreduced diploid oocyte, the result of failure to extrude either the first or second polar body, by a haploid spermatozoon (digynic triploidy).

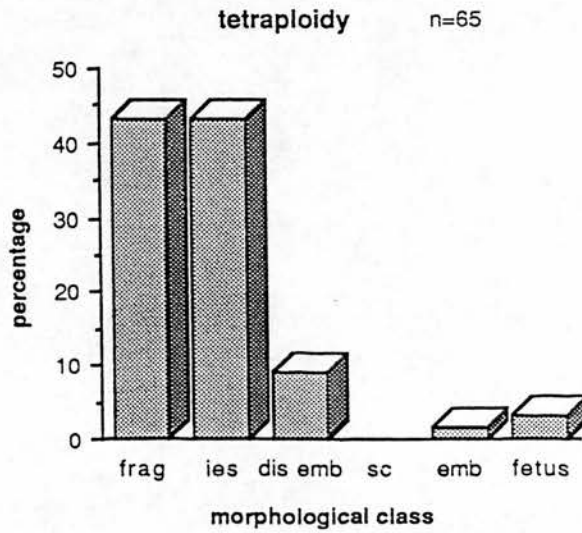
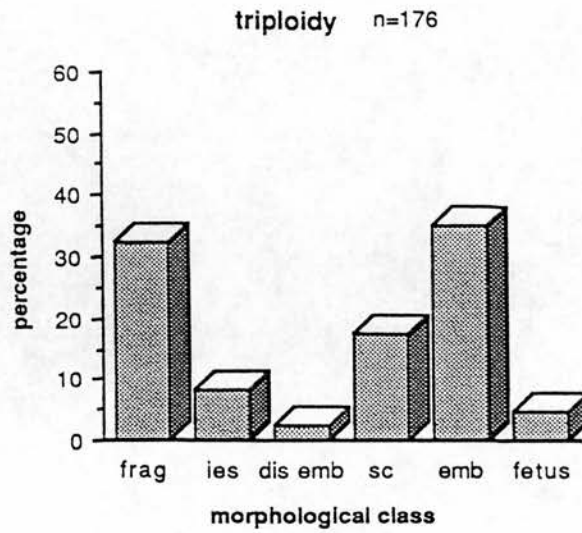
and in animals also (but not yet demonstrated unequivocally in man),

- iv) from the fertilization of a "giant" diploid oocyte by a normal haploid spermatozoon (digynic triploidy) (Austin, 1969; Funaki and Mikamo, 1980; Funaki, 1981).
- v) and the fertilization of a diploid primary oocyte as occurs in LT/Sv strain mice (digynic triploidy) (Kaufman and Howlett, 1986; O'Neill and Kaufman, 1987b).

In man, the most common mechanism of triploidy is believed to be that of dispermy (66%), followed by the fertilization of a haploid oocyte by a diploid spermatozoon, the result of failure of the first or second meiotic division in the male (24%), and finally 10% of triploid conceptions are the result of the fertilization of an unreduced diploid egg by a haploid spermatozoon (Jacobs et al., 1978). While 0.5-1% of all spermatozoa present in man may be diploid (Sumner, 1971; Carothers and Beatty, 1975), they are rarer in mice, and probably do not play a significant role in the incidence of triploidy (Krzanowska, 1974). It has therefore been established that approximately 85-90% of all human triploids are diandric in origin and the rest are digynic (Jacobs et al., 1982). In animals, the proportionate incidence of the type of triploidy encountered, whether diandric or digynic, is generally very species specific (see Dyban and Baranov, 1987), but in certain strains of mice no obvious selection appears to exist (Donahue, 1972a, b).

#### *Clinical Details Of Triploidy In The Human*

Triploidy is generally lethal during the embryonic or early fetal period in mammals. In humans, triploids are commonly spontaneously aborted in the first trimester, usually being presented in abortuses as a well developed embryo (see Figure 1.2). They can, however, occasionally survive to term and rarely a triploid child will live for a few months (Sherard et al., 1986). Long term development, however, is suggestive of diploid/triploid mosaicism. Triploid abortuses exhibit extensive abnormalities involving the heart, the genital and urinary systems, the central nervous system and frequently the limbs, though no consistent syndrome complex has so far been reported (Niebuhr, 1974; Gosden et al., 1976; Harris et al., 1981; Doshi et al., 1983; Wilson et al., 1988). Cell size appears to be enlarged to a certain extent in the definitive red blood cells of both triploid fetuses (Fisk et al., 1989) and neonates (Pai et al., 1982; Sadowitz et al., 1984). Although diploid/triploid mosaicism is compatible with livebirth, the abnormalities associated with these mosaic individuals are similar to those found in pure triploids, but less severe. In older patients, asymmetry of the body and head occurs when one side of the body is less developed than the other, and mental retardation is usually described (see Niebuhr, 1974; Doshi et al., 1983).



**Figure 1.2**

Distribution (percent) of morphological class of triploid and tetraploid abortuses.

**KEY:**

frag (fragments of placenta)

ies (intact empty sac)

dis emb (small disorganised embryo)

(from Warburton et al.,1991).

sc (sac with cord but no embryo)

emb (embryo 10-30mm)

fetus (fetus > 30mm)

The gestational age of triploid abortuses and the phenotypic expression of their membranes has appeared to vary according to the parental origin of the extra haploid set of chromosomes (Hassold et al., 1980; Harris et al., 1981). Diandric triploids have been reported to survive longer than digynic triploids with a mean gestational age of approximately 124 days, while digynic triploids have a mean gestational age of between 69-94 days. Jacobs et al. (1982) found that the mean gestational age of triploid abortuses, in fact, correlated with the development of the partial hydatidiform mole. Hydatidiform changes in the chorionic villi is the most consistent feature of triploidy and one that enables one to distinguish between diandric and digynic triploidy (Harris et al., 1981).

### ***Partial Hydatidiform Moles***

The relationship between triploidy and hydatidiform changes in the chorionic villi has been known for some time (Makino et al., 1964; Carr, 1969), and it is evident that partial moles almost invariably contain two paternal haploid complements and one maternal haploid complement (Szulman and Surti, 1978). They are usually dispermic in origin and have a 69 XXY or 69 XXX chromosome constitution. Partial hydatidiform moles may show the presence of hydropic changes in a few, or many, of the chorionic villi, and trophoblastic proliferation is minimal. Some of the villi may contain fetal blood vessels within which primitive red blood cells may be found. Often an embryo is associated with this type of mole, but it is usually dead at the time of diagnosis (Kaufman, 1988). Generally, this class of mole is not associated with a malignant transformation but it is occasionally seen (Gardner & Lage, 1992). The digynic class of triploids, however, rarely shows these hydatidiform changes. In fact, Jacobs et al. (1982) observed that in a study of 106 triploid abortuses all paternally-derived triploids were partial moles whereas only 3 out of 15 maternally-derived triploids were molar.

### ***Sex-Chromosome Constitution Of Human Triploids***

The theoretically possible sex-chromosome combinations that may be encountered in diandric triploid embryos, assuming an equal probability of an X or Y spermatozoon fertilizing an egg, are XXX, XXY and XYY, and for digynic triploid embryos XXX and XXY. A 1:1 sex-ratio is

usually found in digynic triploids, but the sex-ratio of diandric triploids consistently differs significantly from the expected ratio of 1:2:1. Kajii and Niikawa (1977), for example, reviewed 387 triploid abortions described in the literature and found a ratio of 148 XXX: 228 XXY : 11 XYY. Jacobs et al. (1982) found the sex-chromosome ratio in 41 spontaneous abortions arising from dispermy to be 15 XXX: 26 XXY with no evidence of the expected XYY group. Boué et al. (1985) summarised a number of cytogenetic studies on triploid abortuses which indicated a sex-chromosome ratio of 162 XXX: 229 XXY: 10 XYY. Evidently, the XYY group of embryos is encountered much less frequently than the other groups of recognised abortuses, and it has been suggested that they are probably aborted much earlier in gestation (Beatty, 1978). It is unclear, however, whether this is a result of the lethality of the XYY group, or maternal rejection. Boué et al. (1975) have suggested that poor growth in culture of these cells may lead to a failure to report this condition. XXX and XXY triploids (whether diandric or digynic in origin) appear to have, however, a comparable viability in the postimplantation period.

An analysis of the X inactivation status of triploid human abortuses reveals that either one or two X chromosomes may be inactive. Of 27 triploid abortuses that had a XXY sex-chromosome constitution, the cells of 14 abortuses had both X chromosomes active, 2 had one active X, and 11 had two populations of cells, with either one or two active X chromosomes. Among 9 XXX triploid abortuses, 4 had two active X chromosomes present and the others had a mixed population of either two or one active X chromosomes present (Jacobs et al., 1979).

#### 1.6.2 TETRAPLOIDY

Tetraploidy accounts for about 5% of all spontaneous abortions in humans. It occurs most commonly by two routes:

- i) inhibition of the first cleavage mitosis, following monospermic fertilization of a normal haploid oocyte (homozygous tetraploidy).
- ii) trispermic fertilization of a normal haploid oocyte (triandric heterozygous tetraploidy).

While the first route is the more common of the two, triandric tetraploids have been recorded in human spontaneous abortions (Sheppard et al., 1982; Surti et al., 1986). Trigynic tetraploids have so far not been reported in human spontaneous abortions.

### *Clinical Details Of Tetraploidy In Humans*

Little is known of the effect tetraploidy has on development because of the small number of embryos/fetuses that have been available to examine. Most tetraploid abortuses are presented as intact or ruptured empty sacs (Carr, 1971b, see Figure 1.2). However, a total of 7 pure tetraploids have reached term to date (Pajares et al., 1990), and tetraploid infants have been known to survive for up to 2 years (Lafer and Neu, 1988). Their abnormalities include microcephaly, cleft palate, microphthalmia and limb defects but no specific tetraploidy syndrome has so far been established (Pajares et al., 1990). An analysis of 120 human tetraploid abortions revealed the sex ratio to be 75 XXXX: 45 XXYY which differed from the expected ratio of 1 XXXX:1 XXYY (reviewed by Sheppard et al., 1982). Like diandric triploid abortuses, the placental tissue of triandric tetraploid abortuses have a predisposition to undergo hydatidiform transformation (Surti et al., 1986). Tetraploid/diploid mosaics are compatible with live birth, and exhibit a wide range of congenital abnormalities and a severe degree of intrauterine and extrauterine growth failure (Kohn et al., 1967; Wilson et al., 1988).

### 1.6.3 ANDROGENESIS

The most common example of androgenesis in man is found in the clinical condition of "complete" hydatidiform mole. Complete moles have an exclusively paternally-derived diploid genome and the majority (92-96%) of complete moles have a 46 XX sex-chromosome constitution (Kajii and Ohama, 1977; Jacobs et al., 1980). This may result from the fertilization of an ovulated egg by a spermatozoon with subsequent duplication of its sperm-derived genome, or this could result from fertilization by a diploid spermatozoon. In the absence of the maternal genome, such embryos would be androgenetic. The remaining proportion of complete moles are 46 XY, and heterozygous since they result from the fertilization of an egg by both an X and Y-bearing spermatozoon (Ohama et al., 1981; Surti et al., 1982), and these



appear to have a greater tendency for malignant transformation. 46 YY moles have not so far been reported and it is thought that these conceptuses fail to develop past a few cleavage divisions since the presence of at least one X chromosome is believed to be essential for early mammalian development (Burgoyne and Biggers, 1976). Complete moles are characterised by the absence of a fetus and its associated membranes, relatively uniform hydropic changes in the chorionic villi and also a marked proliferation of the trophoblast.

#### 1.6.4 PARTHENOGENESIS

No unequivocal cases of parthenogenetic embryos have been recorded in human spontaneous abortions but they are unlikely to be found this late in gestation. Benign and malignant ovarian teratomas, however, are parthenogenetic (Linder et al., 1975), and their development begins with the spontaneous activation of follicles which have completed the first meiotic division and diploidise by suppression of the second meiotic division. In the LT/Sv strain of mice, a high incidence of spontaneous parthenogenesis occurs in both ovarian and newly ovulated oocytes after meiotic maturation (Stevens and Varnum, 1974; Stevens, 1975; Eppig et al., 1977). These embryos occasionally reach the egg cylinder stage, but then become disorganised, and undifferentiated cells migrate away from their site of origin and occasionally become teratomas.

#### 1.7 AETIOLOGY OF POLYPLOIDY IN MAN

Unlike aneuploidy, polyploidy in human spontaneous abortions is not associated with increased maternal age (Carr, 1971a; Hassold and Chiu, 1985), nor is its incidence increased with the exposure to spindle-disrupting agents. The degree of maturation of the egg, however, may be of considerable importance in the aetiology of polyploidy, since animal experiments have shown that abnormal fertilization and alterations in egg ultrastructure occurs with ageing.

### 1.7.1 POSTOVULATORY AGEING OF THE EGG AS A CAUSE OF POLYPLOIDY IN

#### MAN.

In all species so far studied, the recently ovulated egg has a number of effective defence mechanisms to prevent polyspermic fertilization. Normal fertilization involves the successful penetration of the ovulated egg by a spermatozoon, and results in an elevation of intracellular  $\text{Ca}^{2+}$  followed by regular transient oscillations in  $\text{Ca}^{2+}$ . This is believed to signal the initiation of development with completion of the second meiotic division in the egg (Cuthbertson et al., 1981; Cuthbertson and Cobbold, 1985; Miyazaki, 1988; Kline and Kline, 1992). In the optimum situation for fertilization, the number of spermatozoa that reach the egg within the oviduct is orders of magnitude less than the number found in the ejaculate (Braden and Austin, 1954a), thereby reducing the chances of polyspermy. In recently ovulated eggs, the first spermatozoon that successfully penetrates the zona pellucida and makes contact with the vitelline membrane of the egg, induces a change in its electrical properties, preventing the fertilization of the egg by other spermatozoa. This is known as the "fast block to polyspermy" (Sato, 1979). Shortly thereafter, cortical granules, present at the periphery of the cytoplasm of the egg, rupture and their contents (i.e proteolytic enzymes) are extruded into the perivitelline space. Their contents interact with the zona pellucida, altering its molecular properties, possibly through the inactivation or removal of species-specific receptor sites for spermatozoa. This cortical reaction represents the "slow block to polyspermy" and prevents further spermatozoa from penetrating the zona pellucida (Gulyas, 1980). While this is known to occur in rats and mice, these mechanisms are not universal. In hamsters, for example, the primary block to polyspermy is restricted to the zona pellucida.

In the absence of spermatozoa in the female reproductive tract, the unfertilized egg ages and irreversible changes occur within it which render these defence mechanisms inadequate. Ultrastructural analyses of mouse, rabbit and hamster eggs have revealed that reorganisation of the cytocortex (Webb et al., 1986), changes in the position of the meiotic spindle and cortical granules all occur with postovulatory ageing (Szöllösi, 1971,1975; Longo, 1980). In recently ovulated, unfertilized mouse eggs, an actin-rich area is believed to overly the meiotic spindle at

the periphery of the egg. In older eggs (18-24 hours postovulatory), the cortical actin becomes evenly distributed and this is believed to be responsible for the rotation of the spindle apparatus from a paratangential to a radial position and for its subsequent migration towards the centre of the egg. The cortical granules, which are involved in the slow block to polyspermy, are unstable and short-lived, and it has been observed that the number of cortical granules in the cortex is inversely proportional to the age of the egg. In rat, rabbit and hamster eggs, the distribution of cortical granules remains the same for the first 24 hours after ovulation. With further ageing, however, the cortical granules appear to be within cytoplasmic blebs in the perivitelline space (Longo, 1974). In the mouse, after about 14-18 hours of ageing in the oviduct, the cortical granules are found to be migrating away from the periphery, into the interior of the egg. After 24 hours, they become swollen, and their contents are dispersed in the cytoplasm (Szöllösi, 1971, 1975).

Experiments with animals have also shown that when fertilization is delayed, the incidence of abnormal developmental pathways increases. Experiments with rats showed that delaying mating until the end of oestrous resulted in up to an 11-fold increase in the incidence of trinuclear eggs which was attributed to dispermy (Odor and Blandau, 1956). Similarly, Hancock (1959) found that when mating in pigs took place up to 48 hours after the onset of oestrus, the incidence of trinuclear eggs increased to about 40%, which was also the result of dispermy. Similar results were reported in rabbits after delayed fertilization (Shaver and Carr, 1967) and delayed insemination (Austin, 1960). Braden and Austin (1954b) curiously detected no detrimental effect of delayed mating or delayed fertilization in mice. Marston and Chang (1964), however, found that delayed insemination in mice resulted in an increase in androgenetic, immediate cleavage embryos, and eggs that had retained the second polar body (digynic), but no increase in polyspermic eggs was observed. A 9-fold increase in triploidy, specifically digyny, was observed with delayed mating in mice by Vickers (1969), while delayed insemination apparently produced a rise in dispermy (Boerjan and De Boer, 1990). In the golden hamster, a delay in mating for up to 9 hours after ovulation results in triploidy in about 5% of preimplantation embryos (Yamamoto and Ingalls, 1972), and up to 5% in 9-day

embryos compared to controls (Yamamoto and Ingalls, 1972; Adachi and Ingalls, 1976). In these studies, triploidy was believed to result from second polar body retention on the basis that their cytogenetic analyses did not reveal any XYY triploid embryos. The absence of this group in 3-day and 9-day embryos, however, is not always indicative of digyny, since XYY embryos might not have survived past the early cleavage divisions.

Postovulatory ageing of the egg, which occurs as a result of delayed fertilization, has also been reported to predispose it to parthenogenetic activation (Donahue and Karp, 1973; Kaufman, 1973a, 1983; Plachot et al., 1985). It is well known that recently ovulated eggs activate less readily than postovulatory aged eggs (Kaufman, 1973a; Webb et al., 1986; O'Neill and Kaufman, 1988; Shaw and Trounson, 1989). Electrical activation studies suggest that this may be a result of postovulatory aged eggs becoming more responsive to intracellular  $\text{Ca}^{2+}$  than recently ovulated eggs (Fissore and Robl, 1992). Postovulatory ageing of the egg *in vitro*, however, does not predispose the eggs to activate as readily as *in vivo*, and it is hypothesised that this may be the result of some oviductal factor absent *in vitro* (Kaufman, 1983).

*In vitro* fertilization (IVF) studies have also suggested that the degree of maturation of the egg can influence not only the success of fertilization, but also the incidence of polyploidy. Most human IVF programs employ preovulatory eggs as a source of female gametes and allow them to mature *in vitro*. Some eggs that are matured for longer than necessary have been observed, on fertilization, to be trinuclear. Some authorities have suggested that these embryos are the result of failure of the egg to extrude the second polar body (Rudak et al., 1985), while others believe them to be the result of dispermy (Santalo et al., 1986).

Animal IVF experiments have supported the findings of human IVF studies. Badenas et al. (1989), for example, found that ageing ovulated mouse eggs for up to 6 hours *in vitro*, results in a significant increase in the incidence of polyploidy after fertilization of these eggs (control: 14.6%, 6 hour aged: 31.6% (the high incidence of polyploidy in the control group is probably a consequence of fertilization *in vitro*)). The cytogenetic analysis of the triploid embryos

demonstrated that the origin of triploidy was dispermy. Furthermore, ageing of the egg for up to 12 hours *in vitro* can also result in lowered fertilization rates (Santalo et al., 1987; Badenas et al., 1989) and to a greater extent in cumulus-free eggs (Gianfortoni and Gulyas, 1985). In contrast, *in vivo* ageing of eggs for 12 hours apparently results in high rates of fertilization (Boerjan and De Boer, 1990).

Interestingly, the fertilization of immature human eggs which occasionally occurs in IVF studies can result in an increase in polyploidy (usually triploidy) as a result of polyspermy (Wentz et al., 1983; Rudak et al., 1985; Van Der Ven et al., 1985). Trounson et al. (1982) believe that this is probably a consequence of cytoplasmic immaturity, resulting in an inadequate cortical reaction through incomplete migration of the cortical granules to the periphery of the cytoplasm. Preincubation of the oocytes for several hours can lead to a reduction in polyspermy through cytoplasmic maturation.

#### 1.7.2 PREOVULATORY OVERRIPENESS AS A CAUSE OF POLYPLOIDY IN MAN

In rats, a delay in the midcycle surge of luteinizing hormone can be experimentally induced with the drug pentobarbital (Butcher and Fugo, 1967; Mikamo and Hamaguchi, 1975). The delay in ovulation resulting from this treatment is believed to cause the eggs to "age" in the germinal vesicle stage of maturation (Freeman et al., 1970). Subsequent fertilization of these eggs results in a striking increase in the frequency of triploidy as a result of dispermy, which is believed to be a consequence of an incomplete barrier to polyspermy. It is difficult to see whether this resulted from an "immature" cortical reaction or was due to changes associated with ageing within the egg, rendering the cortical reaction inadequate (Mikamo and Hamaguchi, 1975). The significance of preovulatory overripeness in humans is still unclear, since a delay in ovulation is difficult to establish.

### 1.7.3 AGEING OF MALE GAMETES AS A CAUSE OF POLYPLOIDY IN MAN

Experiments have suggested that the length of time that spermatozoa reside in the female reproductive tract (or male, a consequence of sexual rest) in mice is not important in the aetiology of polyploidy (Munne and Estop, 1991; Sapp and Martin- DeLeon, 1992), but may influence the fertilization rate in these animals. After ageing of spermatozoa for 48 hours *in vitro*, only 4.4% of eggs were successfully fertilized compared to about 75% in the controls (Munne and Estop, 1991).

### 1.7.4 SUPEROVULATION AS A CAUSE OF POLYPLOIDY IN MAN

Hormonal stimulation is believed to induce changes in the properties of the zona pellucida and vitelline membrane which render them less able to block polyspermy (Maudlin and Fraser, 1977). These authors demonstrated that there was a dose-related increase in diandric triploidy in TO mice after hormonal stimulation with Pregnant Mares' Serum Gonadotrophin (PMSG), while Human Chorionic Gonadotrophin (hCG) stimulation is believed not to affect the chromosome constitution of rabbit embryos (Shaver, 1970). A significant increase in the frequency of digynic triploidy in superovulated female mice of the A/He strain and LT/Sv strain compared to untreated mice has been reported (Takagi, 1970; Takagi and Sasaki, 1976; Speirs and Kaufman, 1988). In humans, Carr (1971a) reported an increase in the number of spontaneous triploid abortions in women who conceived within 6 months of ceasing oral contraceptives, but this finding was not confirmed in later observations by others (Alberman et al., 1980; Boue et al., 1975). Specific types of hormonal stimulation given to women attending IVF clinics have been reported to increase the frequency of polyploidy in fertilized embryos (Wentz et al., 1983). However, this finding has also failed to be satisfactorily confirmed since polyspermy is usually increased with *in vitro* fertilization, due to the much higher concentration of spermatozoa utilised than would usually be present *in vivo* (Fraser et al., 1976; Van Der Ven et al., 1985).

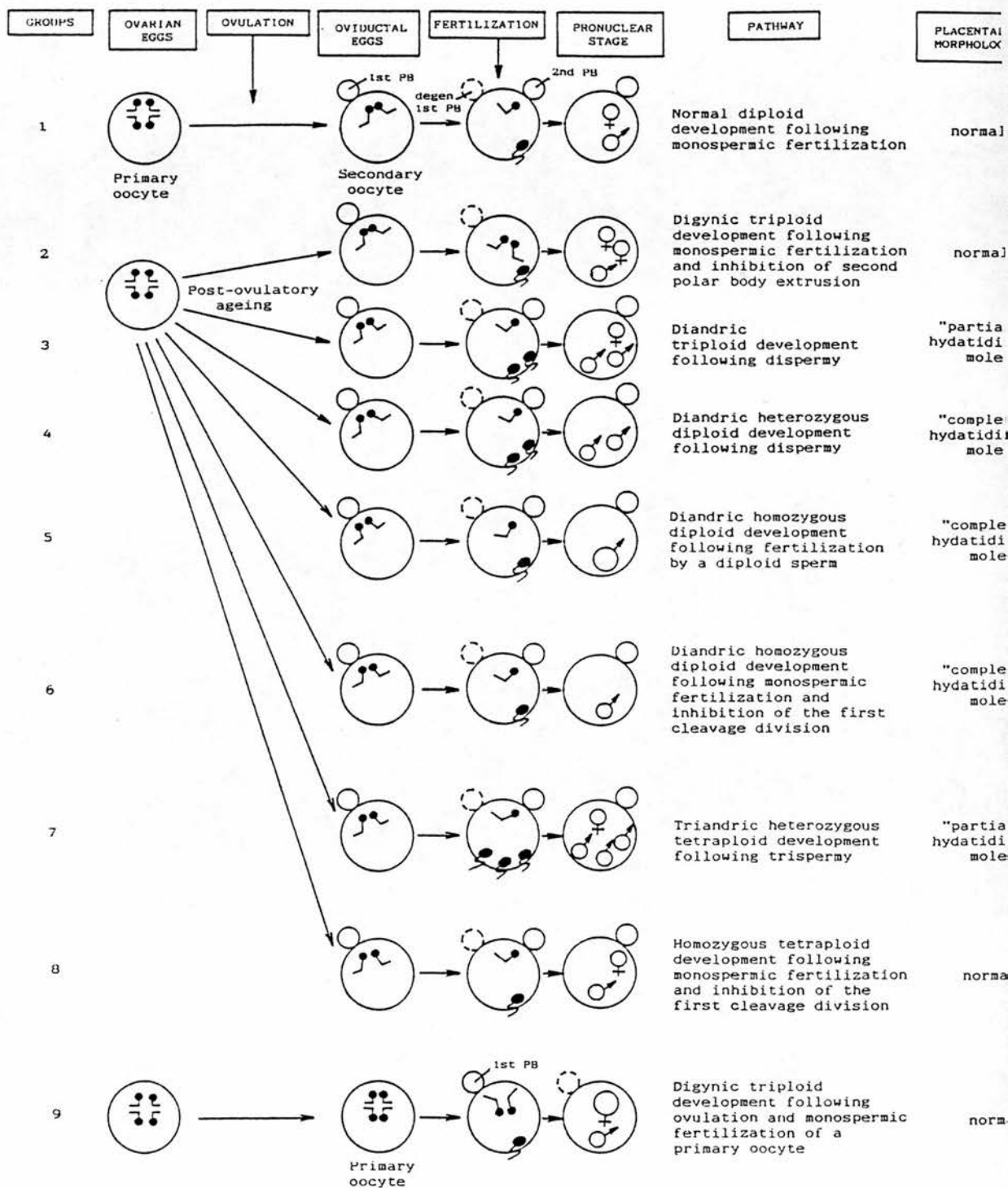


There is an overwhelming weight of evidence to suggest that the degree of maturation of the egg, at the time of fertilization, is primarily responsible for an increase in the incidence of polyploidy. It is clear, that in animals not subjected to experimental conditions, postovulatory ageing of the unfertilized egg, prior to fertilization, rarely occurs. An explanation for this lies in the fact that animals have devised reproductive strategies to ensure that mating only occurs during oestrus, when ovulation is about to occur, and the female is receptive to the male. In humans, however, sexual intercourse occurs at any point in the menstrual cycle and the egg may age considerably in the oviduct before sperm arrive to fertilize it.

### 1.8 HAPLOID, DIPLOID AND POLYPLOID DEVELOPMENT

It has been demonstrated that physical and physiological changes occur within postovulatory aged eggs. It is now possible to speculate on the aetiological origin of the chromosomal abnormalities based on these observations (see Figures 1.3 and 1.4).

The most effective block to polyspermy is provided by the cortical reaction. In postovulatory aged eggs of most species, the cortical granules, which constitute this reaction, can be found to be migrating away from the periphery of the egg towards the centre. If an "inadequate" cortical reaction occurs, the egg may be penetrated by two or more spermatozoa and the consequence of this is most commonly *diandric triploidy* or *triandric tetraploidy*. If on penetration of the zona by one or more sperm, the maternal chromosomes do not take part in subsequent development, androgenetic development may take place. *Homozygous diandric diploidy* can result from the penetration by one spermatozoon with subsequent doubling of its genome (usually by inhibition of the first cleavage division), while *heterozygous diandric diploidy* results from the penetration of the egg by two spermatozoa.



### Figure 1.3

Possible pathways of development and resultant placental morphology (as might be expected in the human, but no equivalent placental histopathology in the mouse) after fertilization of postovulatory aged mammalian oviductal eggs (groups 2-8). Group 1 represents the normal developmental pathway in the absence of postovulatory ageing and following monospermic fertilization, and group 9 represents digynic triploid development following ovulation and monospermic fertilization of a primary oocyte, characteristic of LT/Sv strain female mice (from Kaufman, 1993).

*Digynic triploidy* can result from a change in the position of the meiotic spindle from a paratangential position to a radial one, with subsequent migration of it towards the centre of the egg. This results in the failure to extrude the second polar body on fertilization and thus, two sets of maternal chromosomes are retained within the egg.

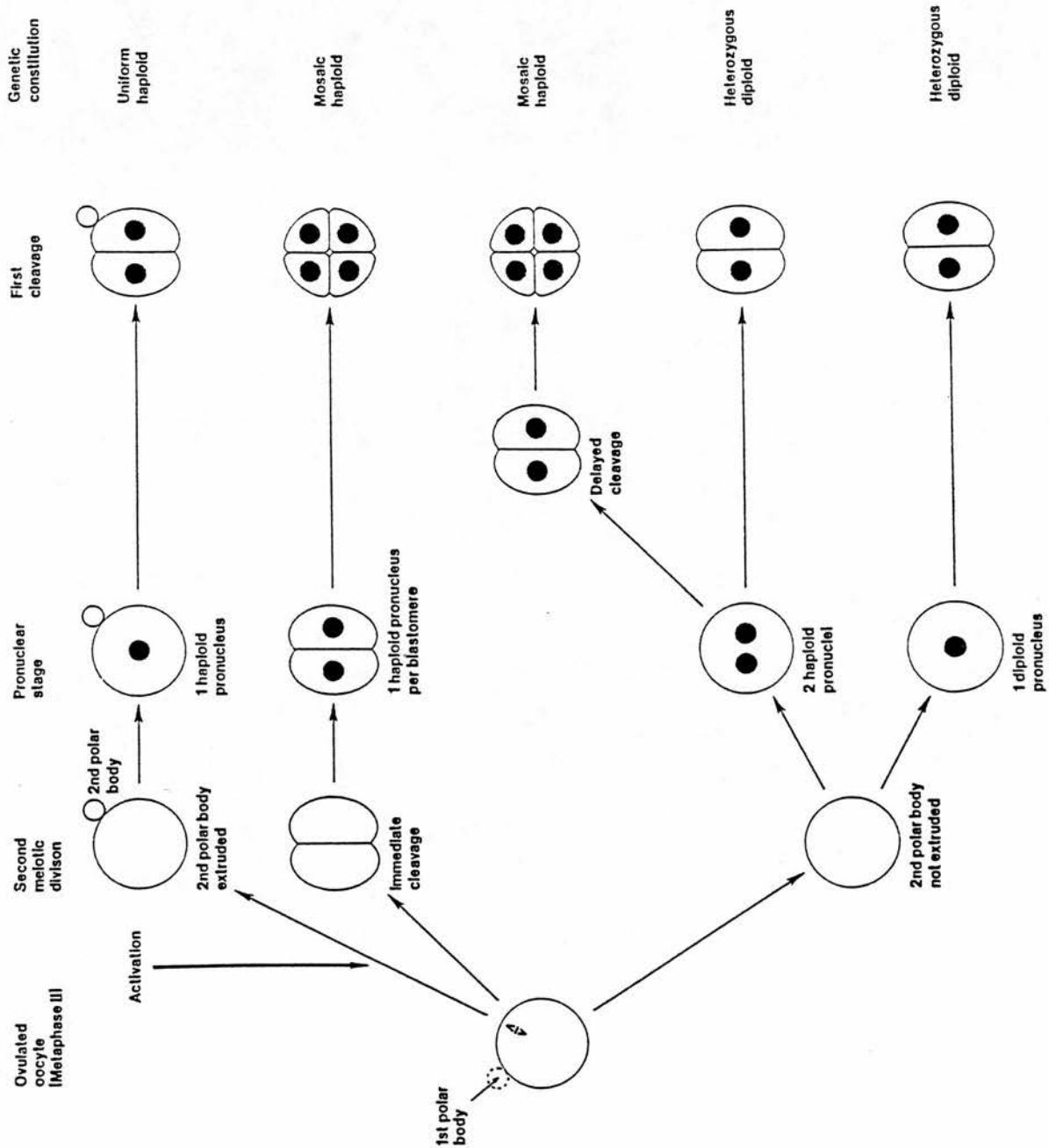
Following the activation of postovulatory aged eggs, the parthenogenetic pathways encountered alter with ageing. The majority of recently ovulated, activated eggs extrude the second polar body upon activation, since the spindle apparatus is still present at the periphery of the egg. The remaining haploid maternal set of chromosomes forms a single pronucleus. Since cells from embryos derived from this type of activated egg are genetically identical, the parthenogenones encountered are *single-pronuclear (homozygous) uniform haploids*.

In postovulatory aged eggs, however, the spindle apparatus is found to be migrating into the centre of the egg. Upon activation, completion of the second meiotic division occurs within the egg, but the extrusion of the second polar body, containing a haploid set of maternal chromosomes, does not occur. Both products of the second meiotic division, retained within the egg, form two single pronuclei, and so *two-pronuclear (diploid) parthenogenones* are observed. These parthenogenones may progress to the first cleavage where the two products will amalgamate to form a diploid embryo which will be heterozygous due to crossing over at meiosis. Alternatively, this two-pronuclear parthenogenone might cleave a few hours after activation, and cells from embryos derived from this *delayed cleavage* group will be haploid and "mosaic" since they possess two genetically dissimilar clones of cells.

Once the spindle apparatus has migrated to the centre of the egg, and on completion of the second meiotic division, an equal cleavage of the egg may immediately occur. Each blastomere will contain a product of the second meiotic division, and cells derived from this type of embryo will be haploid and "mosaic" (as in the case of the delayed cleavage group). At first cleavage mitosis, these *"immediate cleavage"* parthenogenones will, therefore, divide into 4 cells. On some occasions, once the spindle apparatus is central, both products of the second

**Figure 1.4**

Possible pathways of development and genetic constitution of parthenogenetically activated eggs (from Kaufman, 1983).





meiotic division will amalgamate to form a *single diploid pronucleus* which will be heterozygous (assuming that crossing-over has taken place during the first meiotic prophase). (For discussion of the pathways, see Kaufman, 1983).

## **1.9 EXPERIMENTAL INDUCTION OF HAPLOIDY, DIPLOIDY AND POLYPLOIDY IN ANIMALS**

It is important to understand the development of human polyploid embryos since they constitute one of the major groups of chromosomally abnormal spontaneous abortions. Furthermore, some conditions, such as hydatidiform moles, are of clinical importance since they constitute one class of gestational trophoblastic disease.

A great deal of work has been undertaken on the cytogenetic and morphological analysis of human spontaneous polyploid abortuses. However, a proportion of the most lethal conditions have not been available for analysis since they have often been aborted at a much earlier stage of development, and it is impossible to accumulate meaningful, statistical information from the relatively small numbers of human female gametes available for analysis. To examine early events in polyploid development, in a systematic way, a number of techniques have been developed to model them in experimental animals. The experimental induction of haploid, diploid and polyploid embryos will enable an understanding of the fundamental processes of mammalian development and on the roles of the parental genomes. Furthermore, large numbers of animals can be utilised to allow meaningful statistical analysis to be performed.

### **1.9.1 EXPERIMENTAL INDUCTION OF PARTHENOGENESIS**

Parthenogenetic embryos, by definition, develop without any contribution from a male. These embryos differ from gynogenetic embryos, in which spermatozoa act as the activating stimulus, though the male genome is subsequently eliminated and takes no further part in the development of the embryo. Parthenogenetic embryos can be produced in mammals by a variety of means (see Beatty, 1957; Graham, 1974; Whittingham, 1980; Kaufman, 1983, for

review). This has been accomplished both *in vivo* (i.e. within the reproductive tract), and *in vitro*. *In vivo* techniques include the application of heat to the oviducts (Braden and Austin, 1954b, c), electrical stimulation (Tarkowski et al., 1970; Witkowska, 1973a, b; Kaufman et al., 1975), and exposure to anaesthetics and ethanol (Kaufman, 1975; Dyban and Khozhai, 1980). *In vitro* activation methods include a variety of physical and chemical stimuli to oocytes recovered from within the reproductive tract. The postovulatory age of the egg and the culture conditions have also been shown to modify the parthenogenetic pathway observed (Kaufman, 1983).

### 1.9.2 EXPERIMENTAL INDUCTION OF ANDROGENESIS AND GYNOGENESIS

Haploid embryos can be produced successfully by zygote bisection at the 1-cell pronuclear stage (Tarkowski and Rossant, 1976; Tarkowski, 1977). Haploid and diploid androgenones and gynogenones can also be produced using nuclear manipulatory techniques. In the case of haploids, a single pronucleus of a 1-cell fertilized diploid embryo is removed (Modliński, 1975, 1980; Surani et al., 1986). Non-extrusion of the second polar body, or subsequent doubling of the haploid genome at the first cleavage mitosis with cytochalasin B or D, can restore the diploid status (Hoppe and Illmensee, 1977; Markert and Petters, 1977; Modliński, 1980; Borsuk, 1982; Surani and Barton, 1983). Alternatively, a second pronucleus, of similar parental origin, can be inserted into a 1-cell embryo to produce a heterozygous diploid embryo (Barton et al., 1984; Surani et al., 1984; McGrath and Solter, 1984; Surani et al., 1986; Kaufman et al., 1989a). Recently, Kono et al. (1993) reported that androgenetic mouse embryos could also successfully develop from the *in vitro* fertilization of enucleated oocytes.

### 1.9.3 EXPERIMENTAL INDUCTION OF TRIPLOIDY

Digynic triploid embryos are technically easier to produce experimentally than diandric triploid embryos (for review see Beatty, 1957; Niemierko and Opas, 1978; Dyban and Baranov, 1987). Indirect methods of inducing digynic triploids have included superovulation (Takagi, 1970; Takagi and Oshimura, 1973; Takagi and Sasaki, 1976; Maudlin and Fraser, 1977; Speirs and Kaufman, 1988), postovulatory ageing of the egg (Shaver and Carr, 1967;

Vickers, 1969; Yamamoto and Ingalls, 1972), and *in vitro* fertilization (IVF). Triploidy in the latter case is, however, mostly believed to result from polyspermy (Fraser et al., 1976; Wentz et al., 1983; Van Der Ven et al., 1985; Plachot et al., 1985), although in one report, approximately 10% of the triploid embryos obtained following IVF treatment were believed to be digynic in origin (Restagno et al., 1988). In general, however, these techniques are not reliable enough to use in a systematic investigation of triploidy, and more efficient techniques have been devised.

One such technique, is to prevent second polar body extrusion, thus retaining both products of meiosis in the egg, so that, on fertilization, a digynic triploid embryo will be produced. This has been achieved in amphibians (Fankhauser, 1945), and to a lesser extent in mammals (Beatty and Fischberg, 1949; Fischberg and Beatty, 1952), by the application of heat treatment to the oviducts at the time of the second meiotic division, but other chromosomal abnormalities may also result. Cold treatment is also an effective means of producing digynic triploids in amphibians (Fankhauser, 1945), but it is ineffective in mammals (Beatty and Fischberg, 1949). The exposure of fertilized eggs to cytoskeletal inhibitors such as colchicine, colcemid, cytochalasin B or D and phorbol myristate acetate (PMA) can also inhibit second polar body extrusion, if given at the appropriate time (Edwards, 1954, 1958a; Bomsel-Helmreich, 1965; Niemierko, 1975; 1981; Surani and Barton, 1983; Niemierko and Komar, 1985; Speirs and Kaufman, 1989a). Colchicine is very effective in mice and rabbits, but it is toxic to the eggs and both it and colcemid may cause high rates of aneuploidy. Less toxic inducers of triploidy are the cytochalasins and PMA. High rates of triploidy occur when the eggs are fertilized *in vivo* and exposed to these agents *in vitro*. This enables visible confirmation of triploidy before the embryos are transferred to recipient females. Exposing recently fertilized embryos, which have extruded the second polar body, to a strong osmotic shock, can lead to the reincorporation of the second polar body back into the embryo, and in 30-60% of appropriately treated embryos, digynic triploidy may result (Opas, 1977). Digynic triploid embryos can also be produced using nuclear manipulation techniques described by McGrath and Solter (1983). In this case, the female pronucleus from a "donor" 1-cell embryo is transferred to a recipient 1-cell embryo. After fusion of the karyoplast with the cytoplasm of the recipient embryo using,

for example, Sendai virus or more recently electrofusion (Tsunoda et al., 1987; Kono and Tsunoda, 1988), a triploid embryo is produced. Diandric triploid embryos are produced experimentally by nuclear manipulation in the same manner as the digynic triploids except that a male pronucleus, instead of a female pronucleus from a "donor" embryo, is transferred (Kaufman et al., 1989b, c).

#### 1.9.4 EXPERIMENTAL INDUCTION OF TETRAPLOIDY

A variety of techniques have been used to induce tetraploid development. Tetraploidy is usually induced by inhibition of the first cleavage division and the resulting tetraploid embryos are thus homozygous. Beatty and Fischberg (1951, 1952), for example, induced the development of preimplantation tetraploids in mice by circulating hot water around the oviduct at the estimated time of first cleavage, and 12% of the blastocysts recovered were tetraploid. Colchicine-induced suppression of the first cleavage division has also been used with some success (Edwards, 1954, 1958a), and a small number of tetraploid and diploid/tetraploid embryos have resulted. Agents which can induce blastomere fusion such as inactivated Sendai virus (Graham, 1971; O'Neill et al., 1990) and polyethylene glycol (Eglitis, 1980; Eglitis and Wiley, 1981; Spindle, 1981) have been employed to induce tetraploidy, but they have resulted in a very limited degree of postimplantation development. Snow (1973, 1975, 1976) was more successful with the exposure of 2-cell stage mouse embryos to cytochalasin B, which inhibits cytokinesis but not karyokinesis at the second cleavage division. Successful implantation rates of 60% occurred, and advanced postimplantation embryos, including live borns, resulted but similar levels of success have never been repeated either by Snow or by others. Indeed, development beyond implantation has not so far been reliably achieved using this approach. Despite this apparent limitation, much data on the rate of preimplantation tetraploid development has been accumulated using this method of producing tetraploid embryos (Smith and McLaren, 1977; Tarkowski et al., 1977).

Electrofusion has been shown to be an extremely efficient fusigenic stimulus because it is of short duration, elicits a quick reaction, and is highly reproducible. This stimulus is able to fuse adjacent blastomeres at the 2-cell stage, and unlike other methods of inducing tetraploidy, it does not require the blastomeres to be fused to be at a particular stage in their cell cycle. Furthermore, tetraploid embryos produced in this way show no evidence of reverting to diploid/tetraploid mosaicism (James et al., 1992). This experimental approach has previously been applied to rabbit embryos (Ozil and Modliński, 1986), bovine embryos (Iwasaki et al., 1989), as well as to mouse embryos (Kubiak and Tarkowski, 1985; Kato and Tsunoda, 1987; Winkel and Nuccitelli, 1989), though in these studies, only preimplantation development was achieved. These, and more particularly, the recent study by Kaufman and Webb (1990), would seem to indicate that electrofusion *per se* has little or no obvious deleterious effects on preimplantation development, unlike other fusigenic agents, despite the fact that no liveborn tetraploid mouse embryos have been so far reported.

#### 1.10 HAPLOIDY, DIPLOIDY AND POLYPLOIDY IN ANIMALS: INFORMATION FROM SPONTANEOUS AND EXPERIMENTAL INDUCTION

##### 1.10.1 HAPLOID AND DIPLOID DEVELOPMENT

In general, diploid embryos develop better than haploid embryos. Haploid gynogenones, for example, have fewer cells than diploid gynogenones recovered on the 4th or 5th day of gestation and only about 3% achieve the blastocyst stage (Modliński, 1975, 1980; Tarkowski, 1977; Surani et al., 1986). Haploid androgenones have an even poorer developmental potential with only 4% reaching the 4-cell stage (Surani et al., 1986). However, 50% will only contain a Y chromosome and would not be expected to develop for more than a few cleavage divisions (Burgoyne and Biggers, 1976). The poor viability of androgenones may be accounted for by the mechanical trauma to the egg with the removal of the female pronucleus, since it is closely associated with the second polar body and related cytoskeletal system (Modliński, 1975). Recently, Kono et al. (1993) reported that 11% of their haploid mouse androgenones reached



the blastocyst stage. Their method for producing the androgenones involved the *in vitro* fertilization of enucleated oocytes, and it could be speculated that the removal of metaphase II chromosomes for enucleation is less traumatic to the oocyte than pronucleus removal. Haploid parthenogenones have a better developmental potential than haploid androgenones and can reach the blastocyst stage in a high proportion of cases (Kaufman, 1983).

Kaufman (1978), obtained haploid postimplantation development when he activated ovulated eggs *in vitro* and transferred the "immediate cleavage" type embryos to pseudopregnant recipients. On day 9 of gestation, he found 7 egg cylinder stage haploid embryos, but only two were pure haploids, although they did contain healthy dividing cells. Tarkowski et al. (1970) and Witkowska (1973a, b), in contrast, activated ovulated eggs *in vivo* and produced a mixture of haploid and diploid embryos of which a high proportion implanted. The disadvantage of the latter system is that it is impossible to know which haploid developmental pathway was most successful.

The development of diploid gynogenones is consistently better than diploid androgenones. Some 60-80% of diploid gynogenones and a smaller proportion of androgenones can achieve the blastocyst stage (Modliński, 1980; Surani and Barton, 1983; McGrath and Solter, 1984; Surani et al., 1984, 1986; Latham and Solter, 1991). Furthermore, Kono et al. (1993) found that in the case of androgenones, the proportion reaching the blastocyst stage depended on whether the embryo was biparental or the result of diploidisation of the haploid paternal pronucleus. Only a small proportion of gynogenetic and androgenetic embryos implant (Surani and Barton, 1983; Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984; Surani, 1985, 1986). A proportion of diploid gynogenones can develop up to the forelimb bud stage with about 25 pairs of somites on day 10 of gestation. However, they are smaller than genetically normal diploid embryos of a similar developmental age, and appear to have a deficiency of their extra-embryonic membranes (Surani and Barton, 1983; Surani et al., 1984). The postimplantation development of androgenetic (diandric) heterozygous diploid mouse embryos to the egg cylinder and to early somite stages can also occasionally be achieved (Barton et al., 1984;

Kaufman et al., 1989a). The early somite embryos that were recovered appeared small and poorly developed, but they had extensive trophoblastic tissue. Interestingly, Kaufman and his colleagues recovered only XY diandric diploid embryos. This is significantly different from the expected sex-chromosome ratio of 1 XX: 2XY :1 YY. While it is expected that YY embryos will not progress for more than a few cleavage divisions, the absence of XX embryos is more intriguing. This may be the result of abnormal X-inactivation patterns in these embryos, although it has been found that even in  $X_p O$  mouse embryos, the single paternally-derived X remains active (Lyon, 1988). Alternatively, it is possible that a paternally imprinted X chromosome has a retarding effect on early mouse development, as it appears to do so in the postimplantation period (Thornhill and Burgoyne, 1992).

Diploid mouse parthenogenones develop normally during the preimplantation period (Kaufman, 1983), and a high proportion can reach the blastocyst stage. After implantation they have the same developmental fate as diploid gynogenones. This is not species specific, since parthenogenetic rabbit embryos activated with repetitive pulse stimuli, die at a similar stage of postimplantation development as mouse parthenogenones with a comparable range of developmental abnormalities (Ozil, 1990). It was reported that parthenogenetic diploid nuclei, isolated from the inner cell mass region of spontaneously activated LT/Sv parthenogenetic blastocysts, when transferred to enucleated fertilized cytoplasm, can develop into viable young (Hoppe and Illmensee, 1982), but these results have never been repeated and as such must be considered dubious. Subsequent experiments have shown that transplantation of a male and female pronucleus from a fertilized egg into an enucleated activated egg, results in development to term, but the reciprocal transfer of diploid parthenogenetic pronuclei into an enucleated fertilized egg does not (Mann and Lovell-Badge, 1984).

Studies with mice, using the available reconstruction techniques (see Surani, 1985, 1986; Solter, 1988 for review) and also genetic studies (see Cattanaach, 1986 for review), demonstrate that the maternal and paternal pronuclei have complementary roles during development, and



that both are needed for development to term. Furthermore, both genomes must be present within the one cell for development to proceed normally. The phenotypes of androgenones are quite different from gynogenones and parthenogenones, and it is now generally believed that the paternal genome is required for trophoblast development and that the maternal genome is required for the development of the embryo proper (Barton et al., 1984, 1985; Surani et al., 1984; Surani, 1984, 1985). Aggregation chimeric experiments have shown that androgenetic cells can be "rescued" if combined with normal diploid cells but they only become located in the yolk sac and trophoblast, and not in the embryo proper (Surani et al., 1988), even though some androgenetic cells can be found in the inner cell mass at the blastocyst stage (Thomson and Solter, 1989). However, by injecting an androgenetic inner cell mass into a fertilized blastocyst, liveborn chimeric mice can result with substantial contributions of the injected cells to the embryo proper (Barton et al., 1991).

Aggregating parthenogenetic or gynogenetic cells with normal diploid cells can also "rescue" them. Such studies have shown that parthenogenetic cells can contribute extensively to the resultant embryo, including the brain and germ cells (Stevens et al., 1977; Surani et al., 1977; Stevens, 1978; Anderegg and Markert, 1986; Fundele et al., 1989, 1990; Nagy et al., 1987, 1989). Aggregating androgenetic and parthenogenetic embryos, however, does not result in normal development and the resultant embryos are resorbed by day 15 of gestation (Surani et al., 1987).

#### 1.10.2 POLYPLOID PREIMPLANTATION DEVELOPMENT

##### *Triploidy*

Most of the information available on mammalian triploid development has arisen from studies on various strains of mice. Early experiments on triploidy in amphibians, demonstrated that it often caused a delay in the onset of metamorphosis but that the general rate of development of triploid cells was normal or nearly normal (Fankhauser, 1945). Triploid mouse embryos are,

however, believed to have 20-30% less cells than diploid embryos after 3.5 days of cleavage (Beatty and Fischberg, 1951). Takagi and Sasaki (1976) also believed triploid mouse embryos to have a cell cycle time that was about 10% longer than that of normal diploid embryos. These authors hypothesised that, by the tenth cleavage division, the cell number in triploids would be about one-half that of diploids and that after 20 cleavage divisions, their cell number would be one-quarter that of diploids. Edwards (1958b) observed that, while the triploid preimplantation mouse embryos in his study consisted of fewer cells than diploid controls, this was a result of a delay in entering the first cleavage rather than a slower cleavage rate. Baranov (1976) examined the preimplantation development of spontaneous triploids and found that while 8 out of 17 triploid embryos examined had fewer blastomeres than diploid embryos, the cell number in the other triploids did not differ from that of the diploids. He concluded that the sample size was not sufficient to allow any assumptions to be made. Niemierko (1975) believed that any reduction in cell number in the triploids she studied probably resulted from the stress of exposing the eggs to the cytochalasin B treatment to induce triploidy. In rabbits, Bomsel-Helmreich (1965) described how blastocyst diameter in her sample of cytochalasin B induced triploids was consistently smaller than in the diploid embryos, but this information reveals little about triploid embryo cell number and embryo viability. Furthermore, the stress of the treatment could have contributed to this observation. Funaki (1981) concluded on a sample of only 5 "giant" digynic triploids of various developmental ages from the Chinese hamster, that the triploid embryos appeared to develop as well as the diploids.

The number of human preimplantation triploid embryos available for analysis is very small, and information on their development has been derived from observations on "accidental" triploid conceptions from IVF treatments, and from triploid cell lines retained in tissue culture. In some reports, human diandric triploids have apparently been able divide normally for the first few cleavages (Mettler and Michelmann, 1985). In other reports, cleavage is abnormal and frequently the first cleavage division can result in 3 blastomeres (Kola et al., 1987). In tissue culture, triploid cell lines are reported to have a normal cleavage rate (Kuliev et al., 1975; Hassold and Sandison, 1983).

### *Tetraploidy*

Beatty and Fischberg (1951) found that the mean cell number in their small sample of 8 mouse tetraploids was about half that of diploids. This has been a general finding by others examining mouse tetraploid blastocyst cell numbers in comparison to control diploid blastocysts (Snow, 1975, 1976; Smith and McLaren, 1977; Tarkowski et al., 1977; Spindle, 1981; Kubiak and Tarkowski, 1985). In one of these studies (Smith and McLaren, 1977), the preimplantation development of cytochalasin B-induced tetraploid embryos *in vitro* was compared with that of control diploid embryos. It was observed that the mean cell number of cytochalasin-induced tetraploid blastocysts was slightly less than half of that of control diploids incubated under similar conditions. A significant proportion (over 40%) of their tetraploid embryos, and a somewhat smaller proportion (18%) of their controls, however, had died by 60 hours incubation *in vitro*. Any effect of ploidy in this experimental system, therefore, may have been accentuated by the less than optimum culture conditions employed by these and other researchers. Since *in vitro* culture conditions are inevitably suboptimal compared to conditions *in vivo* (see, for example, Bowman and McLaren, 1970; Streffer et al., 1980; Harlow and Quinn, 1982; Bavister, 1988; Evsikov et al., 1990), I believe that any attempt at comparing the influence of ploidy on development should, where possible, take this into account. Furthermore, up to 20% of tetraploid embryos produced by the suppression of the second cleavage division with cytochalasin B may revert to diploid/tetraploid mosaicism (Tarkowski et al., 1977). The cell number of mosaic tetraploids during the preimplantation period is apparently less than diploid embryos but more than that of pure tetraploids (Tarkowski et al., 1977).

A reduction in overall cell numbers present in polyploid embryos/fetuses has been considered to be important in relation both to the morphological abnormalities commonly observed in this group, and their poor viability (see Beatty, 1957). Poor development during this time may lead to failure of the embryos to implant, or they may possess too few cells for normal pattern formation mechanisms to operate during the postimplantation period. It has been reported that the mean number of nuclei in polyploid embryos of the same developmental age is

approximately inversely proportional to the number of chromosome sets present (Beatty and Fischberg, 1951). This relationship, however, has never been systematically examined. The studies indicated above have not been able to rule out suboptimal experimental conditions accounting for their results nor have sufficient numbers been examined to allow statistical validation of their conclusions.

#### 1.10.2 POLYPLOID POSTIMPLANTATION DEVELOPMENT

##### *Triploidy*

In laboratory animals, no pure triploid mammalian embryo has so far reached term, and survival to about midgestation is the norm. This is a situation different to that found in man. A great deal of information on the postimplantation development of digynic triploidy has accumulated from the use of mice which have a high spontaneous incidence of triploidy, such as the LT/Sv strain of mice. Young LT/Sv female mice regularly and spontaneously ovulate 50% of their eggs as primary oocytes (Kaufman and Howlett, 1986; Speirs and Kaufman, 1988), which are capable of being fertilized and give rise to digynic triploid embryos (Kaufman and Speirs, 1987; O'Neill and Kaufman, 1987b). Mating these female mice with males whose genome contain a "marker" chromosome allows the unequivocal cytogenetic confirmation of digynic triploidy. The development of triploids in these mice is optimal because these embryos are not exposed to experimental conditions. Information is sparse on other animals because they only display very low rates of spontaneous triploidy (Wróblewska, 1971; Baranov, 1976).

The principal characteristic of triploids is their developmental retardation compared to diploid embryos. In mice, diandric triploid embryos generally appear to be morphologically normal although smaller than fertilized embryos at similar developmental stages. Advanced embryos usually have about 25-30 pairs of somites and are at the forelimb bud stage by 10 days of gestation (Kaufman, et al., 1989b, c). It is rare to find viable embryos after this time, however recently, a triploid mouse embryo, which was possibly diandric in origin, has been recovered

on day 14 of gestation. On closer examination, however, it was found to be developmentally retarded by about one day (Bos-Mikich & Whittingham, 1992). The extra-embryonic membranes of the diandric triploid embryos do not show hydatidiform molar degeneration and appear to be morphologically normal, though the latter has not, to date, been studied systematically. No other animal species so far studied has shown molar changes either, and it is believed that this phenomenon is probably specific to man and the higher primates.

Successful early postimplantation development of digynic triploid mouse embryos has also been achieved (Vickers, 1969; Baranov, 1976; Opas, 1977; Niemierko, 1981; Surani and Barton, 1983; Kaufman and Speirs, 1987; Kaufman et al., 1989b; Speirs and Kaufman, 1989a, b). Although they can achieve the same developmental stage as the advanced diandric triploids, they are usually recovered at midgestation, only having reached the egg-cylinder stage (Niemierko, 1981), though the degree of development achieved may well be strain specific. They commonly, but not invariably, display various abnormalities, of which the most severe include deformities of the neural tube and heart (Kaufman and Speirs, 1987). They also show a general reduction in body size compared to fertilized diploid embryos of a similar developmental stage and have problems in "turning" (Fischberg and Beatty, 1951; Baranov, 1976; Surani and Barton, 1983; Kaufman and Speirs, 1987). In many strains of mice, however, the 'triploidy syndrome' is seen, where only an empty gestational sac is formed (Wróblewska, 1971). Triploid conceptuses of the A/He and CBA strains of mice, for example, commonly display the triploidy syndrome (Takagi, 1970; Takagi and Oshimura, 1973; Baranov, 1976), but this is uncommon in 129/Sv and its crosses (Wróblewska, 1978). No abnormalities of the extra-embryonic membranes or reduction in their volume has been found in digynic triploids compared to developmentally matched diploid embryos (Kaufman and Speirs, 1987; Kaufman et al., 1989b). Diandric and digynic triploid mouse embryos are also capable of differentiating primordial germ cells (Kaufman et al., 1990). The morphological differences observed between diandric and digynic triploid embryos can only be explained by the origin of the extra haploid

pronucleus. This is probably related to the phenomenon of genomic imprinting, where genes have differential expression patterns depending on the sex of the parent from which they are inherited (Cattanach, 1986).

The information on triploidy in other species is not as extensive, and is very variable. It has been reported, for example, that cytochalasin B-induced triploidy in the rabbit can result in morphologically normal-looking triploid embryos at day 15 of gestation, although they appear to be retarded in growth by about 18 hours. By around day 17, however, no triploid embryos are recovered (Bomsl-Helmreich, 1965; 1971). In the rat, it has been reported that triploid embryos can develop normally up to day 10 of gestation, and some can survive until day 12, but after this time, only diploid/triploid mosaics are recovered (Pikó and Bomsl-Helmreich, 1960).

The sex-chromosome constitution of experimentally-induced and spontaneous triploid mouse embryos may influence their survival as it appears to do in man (Edwards et al., 1967). In a series of 63 diandric triploid mouse embryos recovered on the 10th day of gestation, the sex chromosome ratio of the embryos was 17XXX : 35XXY : 11XYY which is close to the expected ratio of 1:2:1, assuming that X and Y bearing sperm have an equal chance of fertilizing an egg. However, on closer inspection it appeared that none of the most developmentally advanced diandric triploids (25 pairs of somites) were of the XYY type. The embryos of this group were either presomite or at early somite stages (Kaufman et al., 1989c). The limited development of this group has been observed by others (Baranov, 1976; Takagi and Sasaki, 1976). These results can be readily compared to the situation in man where it has been found that in clinically recognised spontaneous abortions very few XYY triploid embryos are encountered (see 1.6.1., and Kajii and Niikawa, 1977; Jacobs et al., 1982; Boué et al., 1985).



Of 120 spontaneous digynic triploid mouse embryos analysed by Speirs and Kaufman (1989b) at day 10 of gestation, the sex-chromosome ratio of the recovered embryos was 60XXX : 60XXY, which was the expected ratio. In digynic triploid mouse embryos from the spontaneously ovulating A/He strain, the observed ratio was 22XXX: 25XXY (Takagi and Sasaki, 1976). Although it changed slightly when females were superovulated, to 30XXX: 45XXY, the results demonstrate that no developmental advantage is gained by a particular sex-chromosome constitution in digynic triploidy, nor does a particular genotype influence their phenotype (Wróblewska, 1971). Apparently in the rabbit, however, preferential survival of digynic triploid XXY embryos has been reported (Bomsl-Helmreich, 1971).

### *Tetraploidy*

Tetraploid mouse embryos, produced by inhibition of the second cleavage division with cytochalasin B, have been claimed to be found alive at birth but did not survive long thereafter (Snow, 1973, 1975, 1976). Only pieces of the liveborns were recovered, and no histological analysis was able to be carried out on these. However, histological examination of 14.5 and 16.5 day tetraploids revealed a wide range of developmental problems involving the blood, lungs, gonads, and brain. In addition, the tetraploid body size was smaller and weighed less than that of comparable diploid embryos. Tarkowski et al. (1977) reported retardation of growth starting as early as day 8 of gestation. Recently, Kaufman and Webb (1990) have produced tetraploid mouse embryos by electrofusion. The most advanced tetraploids, to date, were recovered on day 16 of gestation, but were developmentally equivalent to 14.5 day old diploids (Kaufman, 1991a, 1992a). These embryos consistently displayed craniofacial abnormalities involving the forebrain and eyes. There was also moderate or complete failure of differentiation of the telencephalic vesicles associated with bilateral absence or partial absence of the optic apparatus. In contrast to Snow (1976), abnormalities of the heart and vertebral column have been found in some advanced embryos (Kaufman, 1992a). Furthermore, gonads were normal and able to form primordial germ cells (Kaufman, 1991a). Embryos with a normal vertebral axis typically had a crown-rump length approximately 10-20% smaller than developmentally matched diploid embryos (Snow, 1976; Kaufman, 1991a, 1992a).



Tetraploid rabbit embryos have been reported as being capable of surviving to about day 20 of gestation, but the majority of them apparently die between days 10 and 14 of gestation (Ozil and Modliński, 1986). These embryos appeared to be severely retarded and showed severe malformities of the brain and nervous system although some were apparently normal. Since no histological analysis was performed on them, it is difficult to interpret these findings.

Diploid/tetraploid chimeric mice are capable of advanced postimplantation development and birth, but they commonly display neurological abnormalities and growth retardation. Of 59 chimeras transferred to pseudopregnant recipients in the study of Lu and Markert (1980), only two survived to birth. One was more abnormal than the other, apparently as a consequence of the higher proportion of tetraploid cells present.

The sex-chromosome constitution of homozygous tetraploid embryos up to about the 30 somite stage appears to be close to the predicted ratio of 1 XXXX : 1XXYY (O'Neill et al., 1990; Kaufman, 1991a). Kaufman (1992a), however, found a sex distinction in that 3 out of 4 XXYY homozygous tetraploids possessed certain postcranial vascular abnormalities at 13-14.5 days post coitum (p.c.), while none of the three XXXX homozygous tetraploids possessed these abnormalities.

### **1.11 AETIOLOGY OF ABNORMAL DEVELOPMENT IN POLYPLOIDS**

It is perhaps understandable why pure androgenetic, gynogenetic and parthenogenetic embryos fail to survive to term, since it has been demonstrated that the parental genomes are functionally non-equivalent during development, and both must be present within the same cell, in the appropriate ratio, for successful development to term (Surani and Barton, 1983; Barton et al., 1984, 1985; Mann and Lovell-Badge, 1984; McGrath and Solter, 1984; Surani et al., 1984). The disparity between parental genomes is attributed to a set of imprinted genes whose expression is determined by their parental origin (Cattanach and Kirk, 1985; Cattanach, 1986; Solter, 1988; Surani, 1991).

It is clear, however, that in animals at least, diploid parthenogenones can achieve the same developmental stage as triploid embryos and sometimes also tetraploid embryos despite the fact that parthenogenones only contain maternal chromosomes. The reasons for the abnormal development and premature death in polyploids are clearly complicated and worthy of investigation. While there may be many reasons for the developmental problems in polyploid embryos (e.g. altered ratio of gene products brought about by an imbalance between autosomes and sex chromosomes; for review, see Dyban and Baranov, 1987), factors which may prove to be important in the aetiology of polyploid abnormal development may be embryo cell number and cell size.

For example, in the preimplantation period it has been reported that "the number of cells in polyploid embryos of the same developmental age is inversely proportional to the number of chromosome sets present" (Beatty and Fischberg, 1951). If this is the case, then it is possible that the influence ploidy has on the cleavage rate may be responsible for the overall poor development of polyploids compared to diploid fertilized embryos. Numerous studies, due to their poor experimental design have, however, failed to agree on what influence ploidy has on preimplantation development.

In the postimplantation period, it has been hypothesised that the morphological abnormalities observed in postimplantation tetraploid mouse embryos may be a consequence of an increase in the size of individual cells and a decrease in their overall cell number (Snow, 1975). A relationship between cell and nuclear volume and ploidy has been proposed in relation to mammalian polyploids of *recent* origin. In general, an increase in ploidy is associated with an increase in cell size, though such a relationship is not commonly observed in long established polyploid species, where the cellular and nuclear volume tends to decrease towards that characteristic of the diploid parental species (for review, see Beatty, 1957). An increase in cell size in mammalian polyploids has considerable implications for surface to volume ratios and shape factors (Epstein, 1986).

Earlier work on amphibia found cell or nuclear volume to be directly related to the number of chromosome sets present (Fankhauser, 1945; Fischberg, 1948). While cell size was larger, cell number was proportionately reduced, and the overall body size was about the same or often smaller than that of diploids (Fankhauser, 1945). Cell number reduction occurs in tetraploid amphibia but it appears to have a less dramatic effect on their viability (see Beatty, 1957) compared to mammalian tetraploids. This relationship between cell size and ploidy has not been as fully explored in the higher polyploids of mammals as it has in amphibia, because examples are rarely encountered spontaneously, and only occasionally following experimental manipulation when have they been induced to develop beyond implantation (for recent review, see Dyban and Baranov, 1987). That cell size was increased in tetraploids was noted by Snow (1973, 1975). Thus, yolk sac-derived primitive nucleated blood cells in his tetraploid mouse embryos appeared, on gross inspection, to be four times greater in volume than comparable cells in diploid controls (Snow, 1975). However, no detailed morphometric analyses were performed by him to quantify any relationship which might exist between nuclear and/or cellular volume and ploidy. Mammalian liver parenchymal cells have been observed to exhibit a relationship between cell size and ploidy (Epstein, 1967). Similarly, cloned tetraploid human fibroblasts formed from diploid cells also appear to show a 2:1 relationship between cell size (and nuclear activity) and ploidy (Chang et al., 1983). If such a relationship between cell size and ploidy is proved to exist in mammalian embryos, as it does in amphibia, then this has considerable implications for the number of cells in the embryo, and embryo morphology.

It is of interest, in this respect, to observe the cell number bounds within which genetically normal mammalian embryos can cope with aberrations in cell number. Studies have shown that embryos can regulate their development so that a normal, proportionate animal eventually forms. Tarkowski (1959) showed that destroying one cell of a two- or four-cell mouse embryo gave 11-day embryos of normal size, while Snow demonstrated that killing about 85% of the cells of a 7-day mouse embryo with mitomycin C had a surprisingly limited effect (Snow and Tam, 1979; Snow et al., 1981; Tam and Snow, 1981). The embryos recovered to a very great extent from this gross insult: gross morphology was essentially normal by 13 days p.c and

many of the animals were born when expected. The few obvious signs of the trauma that resulted were a lowered fetal weight, a poor breeding performance and a high incidence of minor axial abnormalities (Gregg and Snow, 1983). Interestingly, aggregating cells at the morula stage, to increase overall embryonic cell number, also results in normal proportionately sized embryos by about midgestation (Tarkowski, 1963; Mintz, 1971; Buehr and McLaren, 1974; Lewis and Rossant, 1982; Rands, 1986a).

Clearly, the explanations for this regulative behaviour are still not fully understood, although it is known that aberrant regulation of imprinted genes concerned with cell growth can result in varying size phenotypes (Cattanach and Kirk, 1985; Cattanach, 1986). The cell-number/cell-size bounds within which embryos can develop normally is important to know because it gives some indication as to how size regulation is achieved and tells us about how pattern formation mechanisms scale to cell size and number. The investigation of cell number and cell size in polyploid embryos provides this opportunity as well as enabling an investigation into the mechanisms underpinning their aberrant development and premature death.

### 1.12 AIMS

The aetiology of abnormal development in polyploids is still unclear. The importance of this condition in human embryonic loss merits its investigation experimentally. In this thesis, I have used the mouse embryo as a model to investigate such aberrant development.

It has been suggested that one of the reasons for such abnormal development might be the influence of ploidy on embryo cell number and cell size. I aim to systematically investigate the influence of ploidy on the cleavage rates of haploid, diploid, triploid and tetraploid preimplantation embryos to determine whether a reduction in cell number occurs with an alteration in ploidy during this time. A deviation from the optimum embryonic cell number

may explain the poor development and failure of these embryos. In addition, it will be possible to determine the influence of parental genomes on development through the investigation of parthenogenetic and diandric and digynic triploid and homozygous tetraploid embryos.

I also wish to examine whether a predictable relationship exists between ploidy and cell size and number in the tissues of postimplantation polyploid embryos. Confirmation of such a relationship may explain the abnormal morphological features encountered in some of these embryos and the premature death of all triploid and tetraploid embryos, and the majority of human embryos with similar genetic abnormalities.

Finally, in order to obtain a more efficient protocol than presently available to investigate the influence of ploidy on development, methodological consideration has been given to the production of parthenogenetic embryos by electrical pulse stimulation. The exposure of ovulated oocytes to pulses of electricity appears to be an effective activating stimulus in this respect and is increasingly widely used. This stimulus is also used in nuclear transplantation studies to fuse either a donor blastomere or nucleoplast fragments to a recipient oocyte and at the same time activate the latter (Stice and Robl, 1988; Collas et al., 1989; Robl and Stice, 1989). Recently this experimental procedure has been used to simulate the intracellular  $\text{Ca}^{2+}$  oscillations, which are known to occur at fertilization. I believe it is important to examine the cytogenetic response of eggs to this stimulus in the light of previous studies on parthenogenetic stimuli (Kaufman, 1982; O'Neill and Kaufman, 1989; O'Neill et al., 1991). There is limited information on this response, and yet this stimulus may be detrimental to normal chromosome segregation and might indicate that electroactivation is unsuitable for further consideration in these types of experiments.

## CHAPTER 2

### MATERIALS AND METHODS



## 2.1 MOUSE STOCKS

All mice were caged in the Medical Faculty's Animal House facilities under a 14 hour (0700-2100h) -10 hour light-dark cycle and received an unlimited supply of pelleted food and water.

### 2.1.1 F1 HYBRID MICE

F1 hybrid female and male mice were obtained through matings between C57BL/6 female x CBA/Ca male mice purchased from Olac. Each breeding pair was allowed to have 6 litters. All littermates of these natural matings were left together until 3 weeks of age when the females were weaned and the majority of males were culled leaving a stock of F1 hybrid female mice. Cages of between 10-12 female mice were left until the mice were 8-12 weeks old before they were used in experiments. A small number of F1 hybrid male mice were used as stud males or were vasectomised and used for matings to produce pseudopregnant F1 hybrid mice for oviduct transfers.

### 2.1.2 LT/Sv STRAIN MICE

A breeding colony of LT/Sv strain mice was maintained from brother/sister matings of the original 6 pairs of LT/Sv mice purchased from the MRC Experimental Embryology and Teratology Unit at Carshalton. Each breeding pair was allowed to have 6 litters. At 3 weeks the majority of the male offspring were culled with the exception of a few which were kept for future breeding stock. LT/Sv strain female mice were used at 8-12 weeks of age.

### 2.1.3 RB(1.3)1BNR STRAIN MICE

A breeding stock of Rb(1.3)1Bnr mice were obtained from the MRC Radiobiology Unit at Harwell and were maintained through brother/sister matings. Male offspring were used as stud males and caged individually, while the female offspring were culled. The genotype of homozygous Rb(1.3)1Bnr males ( $2n=38$ , i.e. the total number of individual chromosomes present) contains two large metacentric chromosomes, being Robertsonian translocations



involving chromosomes 1 and 3 (cf. in normal mice  $2n=40$ ). Consequently, following fertilization by spermatozoa from these males, the paternally-derived haploid genome contains 18 acrocentric and 1 large metacentric "marker" chromosome.

## **2.2 SUPEROVULATION OF FEMALE MICE TO INDUCE OVULATION**

F1 hybrid female mice were induced to ovulate through one intraperitoneal injection of pregnant mares' serum gonadotrophin (PMSG, Intervet) followed 48 hours later by one intraperitoneal injection of human chorionic gonadotrophin (hCG, Intervet). The dosage was 5 international units (IU) of each. PMSG stimulates the follicles in the ovary to develop and mature, and hCG stimulates the mature follicles to ovulate their contents. Ovulation is believed to begin 11 hours after the hCG injection and be virtually complete by 14 hours (Edwards and Gates, 1959). If fertilized embryos were required, each female mouse was caged with one male stud mouse for mating from 1-4 hours after the hCG injection. Mating usually occurred at around 12 hours after the hCG injection. Vaginal plugs were checked for the next morning as evidence of mating.

## **2.3 EVIDENCE OF MATING**

The formation of a vaginal plug was taken as evidence of mating. This was very distinctive in recently mated females and was formed from the coagulation of the males ejaculate in the vagina of the female. The morning of finding this plug was considered the first day of gestation, or if embryonic development was required, this was considered as 0.5 days post-coitum (p.c.).

## **2.4 ROUTINE DISPOSABLE PLASTIC AND GLASS MATERIALS**

All plastics were purchased from Falcon.

#### 2.4.1 TISSUE CULTURE DISHES

Large (60x15mm) and small (35x10mm) round disposable plastic tissue culture dishes were used for dissection and recovery of embryos from the female reproductive tract and for holding microdrops of medium. 24-well multiwell tissue culture dishes were used for the culture of postimplantation embryos and their membranes.

#### 2.4.2 PIPETTES

- 1) The contents of glass Pasteur pipettes were controlled by a small rubber bulb.
- 2) Mouth-controlled micropipettes were used for controlled movement and transfer of preimplantation embryos. These were made from glass Pasteur pipettes which had been pulled over a hot flame and the tapered end broken cleanly at a diameter of approximately 150-200µm.
- 3) 1, 5, and 10ml disposable sterile plastic pipettes were used for moving larger quantities of liquid.
- 4) 1ml glass pipettes

#### 2.4.3 DISPOSABLE SYRINGES

1, 5, 10, 20ml plastic syringes.

#### 2.4.4 PLASTIC TEST TUBES

10ml plastic test tubes used for storing medium.

#### 2.4.5 MICROSCOPE SLIDES

76x26mm glass microscope slides (Chance Proper Ltd.) were routinely used to spread embryos or embryonic tissues for cell and cytogenetic analysis. Larger glass microscope slides (76x39mm) were used in micromanipulatory procedures.

## 2.5 PHOSPHATE BUFFERED SALINE

100ml of concentrated (x10) Dulbecco's Phosphate Buffered Saline (PBS, Gibco) was diluted with 2x distilled water to produce 1 litre of stock solution. This was adjusted to a pH of 7.3 with 0.1 M NaOH. PBS was used as a short term holding medium to recover and isolate embryos from the female reproductive tract. Bovine Serum Albumin (0.4g/100ml ) was added to the PBS to reduce the surface tension of the solution and prevent the embryos and their membranes from sticking to the tissue culture dish and Pasteur pipettes.

## 2.6 MEDIA USED FOR CULTURING PRE- AND POSTIMPLANTATION EMBRYOS

### 2.6.1 PREPARATION OF M16 CULTURE MEDIUM

M16 is a bicarbonate-buffered medium which was used for maintaining the development of preimplantation embryos within the incubator. 100ml of the medium was made up fresh each week and was filtered through sterile 0.22 $\mu$ m Millipore filters into 10ml plastic sterile test tubes and stored at 4<sup>0</sup>C. The medium is based on Whittingham (1971) and 100ml consists of the following:

| <u>compound</u>                      | <u>g/100ml</u>      |
|--------------------------------------|---------------------|
| NaCl                                 | 0.533               |
| KCl                                  | 0.036               |
| CaCl <sub>2</sub> .2H <sub>2</sub> O | 0.025               |
| KH <sub>2</sub> PO <sub>4</sub>      | 0.016               |
| MgSO <sub>4</sub> .7H <sub>2</sub> O | 0.029               |
| NaHCO <sub>3</sub>                   | 0.210               |
| Sodium lactate                       | 0.32ml of 60% syrup |
| Sodium pyruvate                      | 0.004               |
| Glucose                              | 0.100               |
| Bovine Serum Albumin (BSA)           | 0.400               |
| Penicillin G.potassium salt          | 0.006               |
| Streptomycin sulphate                | 0.005               |



|                                       |             |
|---------------------------------------|-------------|
| Phenol Red                            | 0.001       |
| 2x sterile distilled H <sub>2</sub> O | up to 100ml |

## 2.6.2 PREPARATION OF M2 CULTURE MEDIUM

M2 is a HEPES-buffered medium which was used for collecting and handling of preimplantation embryos outside the incubator. 100ml of the medium was made up fresh each week and was filtered through 0.22µm Millipore filters into 10ml plastic sterile test tubes and stored at 4°C. The medium is based on Quinn et al. (1982) and 100ml consists of the following:

| <u>compound</u>                      | <u>g/100ml</u>      |
|--------------------------------------|---------------------|
| NaCl                                 | 0.553               |
| KCl                                  | 0.036               |
| CaCl <sub>2</sub> .2H <sub>2</sub> O | 0.025               |
| KH <sub>2</sub> PO <sub>4</sub>      | 0.016               |
| MgSO <sub>4</sub> .7H <sub>2</sub> O | 0.029               |
| NaHCO <sub>3</sub>                   | 0.035               |
| HEPES                                | 0.497               |
| Sodium lactate                       | 0.32ml of 60% syrup |
| Sodium pyruvate                      | 0.004               |
| Glucose                              | 0.100               |
| Bovine Serum Albumin (BSA)           | 0.400               |
| Penicillin G.potassium salt          | 0.006               |
| Streptomycin sulphate                | 0.005               |
| Phenol Red                           | 0.001               |
| 2x distilled H <sub>2</sub> O        | up to 100 ml        |

The HEPES is adjusted to a pH of 7.4 with 0.1m NaOH to bring the whole medium to the correct pH.

### 2.6.3 PREPARATION OF TISSUE CULTURE MEDIUM 199 FOR POSTIMPLANTATION

#### EMBRYOS

Medium 199 (M199) with Earle's Modified salts (Gibco) is a tissue culture medium used to culture whole embryos and their membranes for cytogenetic analyses. To 100ml of Medium 199 was added 11ml of fetal calf serum (Gibco) and 1ml of colcemid (10µg/ml; Gibco). This medium was kept for several weeks at 4°C. Approximately 1ml of this medium was placed in each well of a 24-multiwell culture dish and was equilibrated in the incubator in an atmosphere of 5% CO<sub>2</sub> in air at 37°C for 30 minutes prior to use.

### 2.7 PREPARATION OF MICRODROPS FOR PREIMPLANTATION WORK

40µl microdrops of the media to be used for preimplantation work were deposited onto the bottom of either a small or large tissue culture dish using a Pasteur pipette. The dish was then flooded with filtered light paraffin oil (BDH) and left in the incubator in an atmosphere of 5%CO<sub>2</sub> in air at 37°C for 30 minutes to equilibrate before being used.

### 2.8 REMOVAL OF CUMULUS CELLS BY HYALURONIDASE

In order to remove attached cumulus cells from recently ovulated eggs or 1-cell stage embryos, hyaluronidase (Sigma) was added to one test tube of filtered M16 culture medium at a concentration of 1mg/ml (760U/ml). Microdrops were set up on large marked tissue culture dishes under filtered light paraffin oil and equilibrated in the incubator for 30 minutes in an atmosphere of 5% CO<sub>2</sub> in air at 37°C.

### 2.9 RECOVERY OF OVULATED CUMULUS MASSES

Recently ovulated cumulus masses containing oocytes (eggs), or recently fertilized 1-cell embryos, were recovered from the ampullary region of the oviduct between 15-25 hours after the hCG injection to induce ovulation. Females were killed by cervical dislocation and laid on their backs on absorbent paper. The fur on the abdomen of the mouse was wetted with ethanol and this kept its hairs out of the dissection, as well as providing a sterile environment. The skin of the mouse was pinched with forceps and a transverse cut made. The skin above and below

the cut was pulled away from it to expose the peritoneum. The peritoneum was pinched with forceps and cut transversely revealing the intestines. The intestines were reflected up and out of the body cavity towards the chest wall and the uterine horns, oviducts and ovaries located. Grasping one uterine horn with watchmaker's forceps, a cut was made with scissors between the ovary and the oviduct, and another cut made lateral to the forceps, between the oviduct and the uterus. Each dissected oviduct was placed in separate discrete drops of PBS on the bottom of a large tissue culture dish.

The oviducts were viewed on a Wild Heerbrugg Dissecting Microscope under low power (x6 objective and x20 eyepieces). The swollen ampullae, containing the cumulus masses, were easily located and a tear was made in the wall of the ampulla by grasping it with watchmaker's forceps and pulling it apart. The pressure within the oviduct is able to push the cumulus mass out into the PBS and the oviduct was then discarded. Using a Pasteur pipette, the cumulus mass, contained within the minimum amount of PBS, was transferred to a previously equilibrated drop of M16 culture medium containing hyaluronidase. After 2-3 minutes, the cumulus cells fell off and the ovulated oocytes or fertilized embryos were transferred with a mouth-controlled pipette through 4 fresh drops of M16 culture medium.

#### **2.10 RECOVERY OF PREIMPLANTATION EMBRYOS FROM WITHIN THE OVIDUCT**

Female mice were killed, as described above, at specific intervals after the HCG injection to recover cumulus-free embryos, of which a high proportion were precompacted/compacted morulae. The oviduct was dissected as described above and placed in drops of PBS + BSA on tissue culture dishes and viewed under a dissecting microscope at low power.

A 1ml disposable syringe attached to a 32-gauge hypodermic needle was filled with PBS + BSA and inserted into the infundibulum of the oviduct (region of the fimbriated os). With practise, the infundibulum was easily located within the coils of oviduct. Using watchmaker's forceps to secure the oviduct within the drop of PBS, the syringe was gently squeezed to flush the fluid through the oviduct and release the embryos into the drop of PBS. It was very



apparent when the oviduct was being properly flushed as it immediately swelled with the contents of the syringe. The oviduct can only be flushed in one direction due to the physiological valve at the utero-tubal junction which prevents backflow. The oviduct was then removed from the drop, and the embryos were transferred, using a mouth-controlled pipette, to a fresh drop of PBS and viewed under high power (x100).

### **2.11 RECOVERY OF PREIMPLANTATION EMBRYOS FROM WITHIN THE UTERUS**

Female mice were killed, as described above, at specific intervals after the hCG injection to induce ovulation. The majority of embryos recovered from the uterus were compacted morulae, zona-intact blastocysts and zona-free blastocysts. The mouse was dissected to expose the uterine horns, oviducts and ovaries. The uterine horn to be flushed was grasped with forceps at one end and gently pulled away from the body cavity. The attached stretched mesometrium running longitudinally to it was cut. To free the uterine horn from the pelvis, a cut was made just above the cervix and another cut made below the junction with the oviduct (in order to avoid the utero-tubal junction which acts like a valve and would hinder proper flushing of the uterus). The dissected uterine horn was rolled on absorbent paper. In doing so, the remaining ragged mesometrium could be trimmed along its length and also excess blood could be removed. This was important because red blood cells in the drop of PBS would hinder viewing of the embryos. Each dissected uterine horn was placed in a separate drop of PBS+BSA on a tissue culture dish and viewed under the low power of the dissecting microscope.

Securing the uterine horn within the drop of PBS, a 1ml disposable syringe attached to a 32-gauge hypodermic needle filled with PBS+BSA was inserted into one end of the horn and gently squeezed to expel its contents. On doing so, the uterine horn swelled with the contents of the syringe and became relatively transparent. The syringe was then inserted into the other end of the uterine horn and flushed again. The embryos recovered were transferred with a mouth-controlled pipette to a fresh drop of PBS+BSA and viewed under the high power objective of a dissecting microscope.

## 2.12 MITOTIC ARREST OF PREIMPLANTATION EMBRYOS IN METAPHASE BY

### COLCEMID

In order that the cells of the embryos that were recovered were arrested in mitotic metaphase for cytogenetic analysis, colcemid (Gibco) was added to M16 culture medium at a concentration of 1µg/ml. Drops were set up on small tissue culture dishes under paraffin oil and equilibrated for 30 minutes in the incubator in an atmosphere of 5% CO<sub>2</sub> in air at 37<sup>0</sup>C. For first cleavage metaphase analysis, 1-cell embryos were left overnight in colcemid and air-dried preparations were made early the following morning (see next section for methodology). In order for the cells in later stage embryos to reach metaphase, precompact /compact morulae were incubated for 4-5 hours and zona-intact and zona-free blastocysts were incubated for 2-3 hours in colcemid after isolation from the female reproductive tract. Air-dried preparations were then made after this time.

## 2.13 AIR-DRIED PREPARATIONS OF PREIMPLANTATION EMBRYOS

Air-dried preparations were made of preimplantation embryos by the method described by Tarkowski (1966) with slight modifications. Only one embryo was spread on each microscope slide. The undersurface of each microscope slide had a small square drawn in the centre with a diamond pencil and was soaked and cleaned in ethanol before use. The embryos to be spread were transferred with a mouth-controlled pipette, in small groups of 2-3, through two discrete drops of 1% tri-sodium citrate (BDH) that were set up on a large tissue culture dish. For first cleavage analysis, the embryos were left for between 5-10 minutes to swell up in this hypotonic solution to ensure adequate spreading of their chromosomes. For later stages, the embryos spent less time in the hypotonic solution because they were liable to lyse. Each embryo was then placed in the centre of the square on a slide with a small amount of sodium citrate solution and a drop of freshly prepared fixative solution, consisting of 3:1 methanol:glacial acetic acid, was dropped onto the embryo. Once the embryo started to spread out, 2 or 3 further drops of the fixative were dropped. The slides were left to air-dry and then were stained with 10% Giemsa nuclear stain (Gurr's improved R66; BDH).

Zona-free blastocysts were much larger and contained many more cells than the other groups of embryos, and so for adequate spreading of these embryos it was found that a much more vigorous swelling of the embryo had to occur. Diluting the sodium citrate solution with distilled water (in the ratio of 1:3 (v/v)) and placing the zona-free blastocysts in it for approximately one minute before placing them on the slides, proved a successful way of spreading these embryos.

## **2.14 RECOVERY OF POSTIMPLANTATION EMBRYOS**

Female mice were killed and dissected to reveal the uterine horns of the reproductive tract. The uteri of pregnant female mice, during late gestation, contain a number of implantation sites along their length. To dissect one uterine horn, one end was grasped near the utero-tubal junction and the stretched mesometrium was cut along its length. A cut was made near the utero-tubal junction and another cut just above the cervix. The dissected uterine horn was rolled on absorbent paper and the ragged mesometrium trimmed along its length. Securing one end with forceps, small sharp scissors were inserted into the wall of the uterine horn at this end and a longitudinal cut was made along the length of the horn. The individual decidua could then be scooped out of the horn with forceps and transferred to a large tissue culture dish full of PBS+BSA.

Under low power of the dissecting microscope, using two pairs of watchmaker's forceps, the embryos and their membranes within the decidua were removed. The extra-embryonic membranes were removed from the embryos and note was taken of stage of development, morphological appearance and, if required, crown-rump length and weight of the embryo. If the embryo was to be cultured along with its membranes (to provide a better sample for an analysis of ploidy), then both embryo and membranes were transferred into one well of a 24-well multiwell tissue culture dish containing M199 culture medium and colcemid. If, however, the embryo was to be processed for wax histology, it was immersed and fixed in Bouin's Fluid and only its membranes were cultured.

### 2.15 BOUIN'S FLUID

Bouin's fluid is a fixative for wax histology and consists of the following:

75ml of Picric Acid (saturated aqueous solution)

25ml of Formalin (40% Formaldehyde)

5ml of Glacial Acetic Acid

Small embryos, of up to 10 days of gestation, were fixed within about 3 hours of immersion into the fixative but for larger embryos, this took about 24 hours. After removal from Bouin's fluid, the embryos were stored in large quantities of 70% ethanol which were regularly changed until the embryos were processed for wax histology. Smaller embryos were routinely sectioned at 7µm while, for larger embryos, the section thickness was 8µm. The sections were stained with haematoxylin and eosin for subsequent histological analysis.

### 2.16 CHROMOSOME PREPARATIONS OF POSTIMPLANTATION EMBRYOS AND MEMBRANES

These preparations are based on a method described by Evans et al. (1972) with slight modifications. After the embryos/extra-embryonic membranes had been cultured for approximately 3 hours, they were transferred to another well in the multiwell culture dish. This well contained about 1ml of a freshly prepared hypotonic solution of 1 part 1.93% tri-sodium citrate and 3 parts 0.56% potassium chloride and the embryos/extra-embryonic membranes were left for 25 minutes in this solution. After this time, they were transferred to another well containing about 1ml of a freshly prepared fixative solution for at least 30 minutes. This fixative solution consisted of one part methanol and 3 parts glacial acetic acid. If the material was stored at 4°C, then the storage time in this solution could be extended to one week.

The material was transferred, with as little fixative as possible, to an embryological watchglass containing about 0.5ml of 60% acetic acid and left for 2-3 minutes. After this time, the material was gently teased apart and taken up in a Pasteur pipette. The solution, containing 60% acetic

acid and disaggregated cells, was deposited as drops on to precleaned unmarked slides that had been heated to 50-70<sup>0</sup>C on a hot plate. One drop was released at a time from the pipette which was held vertically above the slide. Immediately after the drop was deposited on the slide, the majority of it was withdrawn into the pipette again. The cells adhered to the slide at the retreating edge of the fluid and those cells in mitosis were deposited as chromosome spreads. This procedure was carried out all over the slide for a sufficient number of cells in mitosis to be left. With smaller pieces of material, the disaggregation process was carried out directly on the slide to avoid loss of cells. The slides were left to dry and stained with 10% Giemsa nuclear stain.

#### **2.17 STAINING OF SLIDES WITH GIEMSA'S NUCLEAR STAIN**

Slides were stained with a freshly prepared Giemsa's nuclear stain solution (Gurr's improved R66; BDH) at a concentration of 10% in appropriate buffer. Giemsa buffer was prepared from one buffer tablet (BDH) dissolved in 1 litre of distilled water and adjusted to a pH of 6.8. 200 ml of the stain was prepared at a time and filtered through Whatman No 1. paper. Slides were loaded onto a staining rack and stained in a staining bath for 30 minutes. After this time, the slides were rinsed under running tap water and left to dry.

#### **2.18 COUNTING OF THE CELLS IN A PREIMPLANTATION EMBRYO**

Preparations of embryos were analysed under a Leitz Laborlux K microscope. Embryo cell number was counted at a magnification of x250. Unhealthy embryos were discarded after isolation from the female reproductive tract. These embryos were easily identifiable because they were either fragmented, or their cells appeared dark and granular. On some occasions, however, air-dried preparations of some embryos revealed pyknotic nuclei and they were also classed as unhealthy. This may be a consequence of their exposure to colcemid after isolation. Embryos were also discarded and classed as non-analysable if:

- i) their cells were widely scattered about the slide, as this would mean that some cells may have been lost.
- ii) their nuclei overlapped.



Large embryos sometimes contained over a 100 cells, and to accurately count the total number of cells present, a grid was inserted into one eyepiece of the microscope. This enabled the embryo to be divided up into easily countable areas. Cells were counted as one unit if they were in metaphase, prophase, telophase or interphase and two if they were in anaphase.

## **2.19 CYTOGENETIC ANALYSIS OF EMBRYOS AND MEMBRANES**

Embryos were analysed on a Leitz Laborlux K microscope using x100 oil immersion objective lens and x10 eyepieces. The chromosome constitution and ploidy of air-dried embryo preparations was established through the analysis of metaphase spreads. Metaphase spreads in which the chromosomes were dispersed over the slide were not used for establishing the chromosome constitution or ploidy of the embryos.

## **2.20 LOW-ELECTROLYTE SOLUTION**

Low-electrolyte solution was used as a fusion medium and as an activation medium. 50ml was prepared freshly each week and Millipore filtered into small plastic test tubes and stored at 4°C. This solution is based on Kubiak and Tarkowski (1985) and contains:

| <u>compound</u>                  | <u>g/100ml</u> |
|----------------------------------|----------------|
| 0.3M Mannitol                    | 5.466          |
| 0.5% Polyvinyl-Pyrrolidone (PVP) | 0.5            |
| 0.1mM HEPES                      | 0.0024         |

The solution was adjusted to a pH of 7.2 with 0.1 M NaOH.

## **2.21 THE ELECTROACTIVATION OF CUMULUS-FREE OOCYTES**

Superovulated F1 hybrid female mice were killed by cervical dislocation at 15.5, 18.5, 22.5 and 25 hours after the hCG injection (both PMSG and hCG (48 hours later) administered at 1730h, 1430h, 1030h and 0800h). Their oviducts were dissected out and the cumulus masses, containing the ovulated oocytes, were released from the ampullary region of the oviduct into phosphate buffered saline (PBS). The cumulus masses were transferred to microdrops of M16



tissue culture medium containing 1mg/ml of hyaluronidase equilibrated in an atmosphere of 5% CO<sub>2</sub> in air at 37°C for 2-3 minutes to remove the cumulus cells. The cumulus-denuded oocytes were then washed four times in M16.

Batches of 10-12 oocytes were then transferred into a low-electrolyte solution and a similar solution was also present in the electroactivation chamber. The latter consisted of a plastic tissue culture dish which had two platinum wires (Taab Laboratory Equipment Ltd.) fixed on the bottom of the dish with Araldite. Each wire was 250µm in diameter and was fixed parallel to one another, with a space of about 600µm between them. The ends of the platinum wires were connected to a high voltage pulse stimulator model DS7 (Digitimer Ltd.) set at 110 V, 130mA with a pulse duration of 50µsec. The batches of oocytes were placed between the wires and the pulse stimulator was triggered once. The embryos were removed immediately and washed through four drops of M16 medium and returned to the incubator for 5-6 hours.

After this time, four classes of parthenogenone could be determined (see Figure 2.1), namely (a) oocytes which contained a single (haploid) pronucleus, having previously extruded a second polar body (1PN), (b) oocytes which contained two (haploid) pronuclei in the absence of second polar body extrusion (2PN), (c) oocytes which underwent "immediate cleavage" in which two equal-sized blastomeres had formed, each containing a single (haploid) pronucleus, one of the blastomeres representing the second polar body (IC), and (d) oocytes in which a single (diploid) pronucleus developed in the absence of second polar body extrusion (1PND).

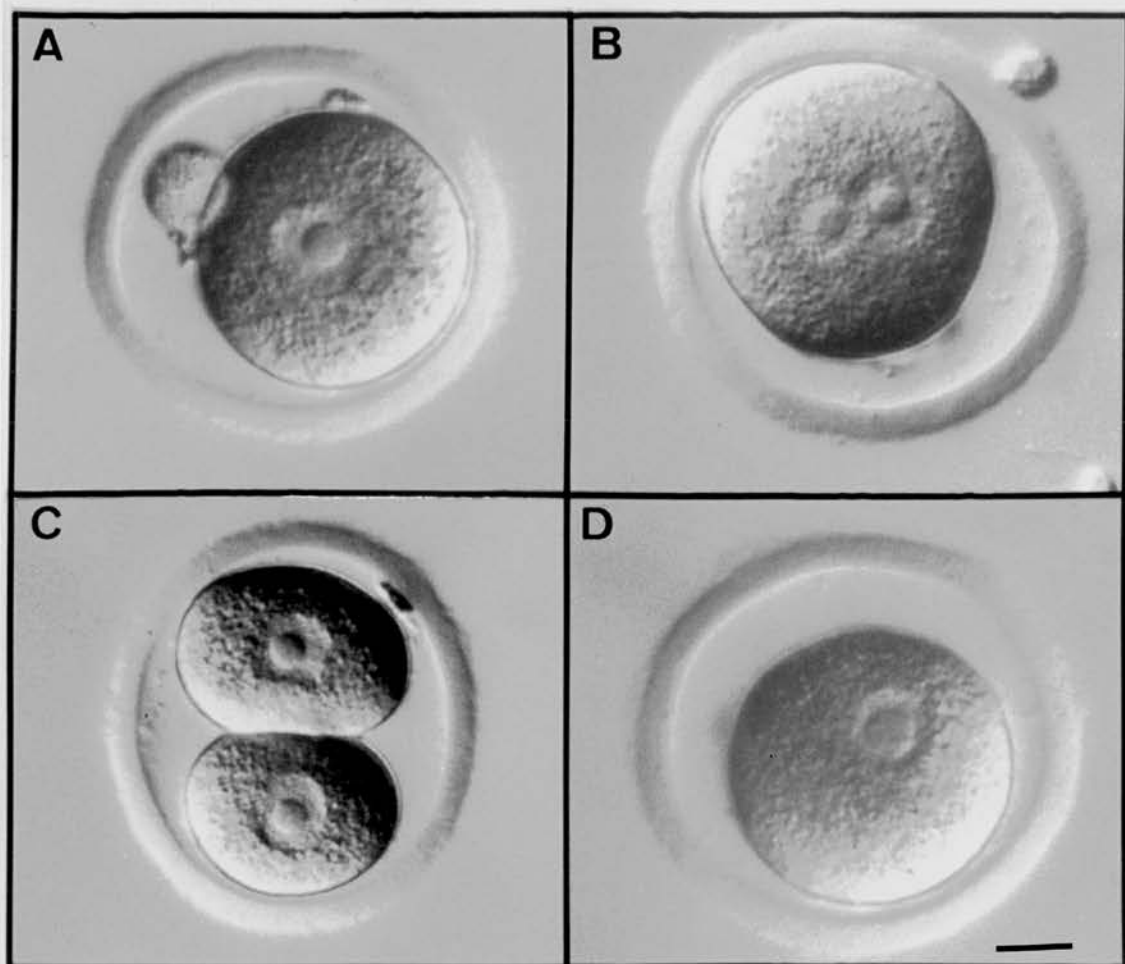
Only the single-pronuclear haploid parthenogenones (1PN) were studied further. They were subsequently transferred 10 hours after activation to microdrops of M16 culture medium containing 1µg/ml of colcemid in order to arrest their development at metaphase of the first cleavage division. Early the next morning, chromosome spreads were prepared by the air-drying technique and stained in 10% Giemsa. The chromosome constitution of each preparation was then determined under an oil immersion objective.

Figure 2.1

The four classes of parthenogenones that can be distinguished after activation of ovulated oocytes (viewed by interference-contrast optics) (from Kaufman, 1983).

- A) Single-pronuclear haploid parthenogenone. Oocyte which contained a single (haploid) pronucleus, having previously extruded a second polar body.
- B) Two-pronuclear diploid parthenogenone. Activated oocyte which contained two (haploid) pronuclei in the absence of second polar body extrusion.
- C) "Immediate cleavage" parthenogenone with two equal-sized blastomeres each containing a single (haploid) pronucleus, one of the blastomeres representing the second polar body.
- D) Single-pronuclear diploid parthenogenone. Activated oocyte in which a single (diploid) pronucleus developed in the absence of second polar body extrusion.

(scale bar = 15 $\mu$ m)



A small proportion of embryos could not be successfully analysed due either to overlapping of the chromosomes in the spread, or scattering of chromosomes, and in a few cases because interphase nuclei were present.

To accommodate for the fact that chromosomes may be lost for technical reasons, and this may inadvertently increase the level of hypohaploid preparations present, an adjusted rate of aneuploidy was used. The formula for this was:

$$\frac{\text{No. hyperhaploid spreads}}{\text{Total No. spreads analysable}} \times 2 = \text{adjusted incidence of aneuploidy}$$

## 2.22 PARTHENOGENETIC ACTIVATION OF OVULATED OOCYTES WITH ETHANOL

Superovulated females were autopsied at 19-19.5 hours after the hCG injection (PMSG and hCG administered at 1400h). Their oviducts were dissected out and the individual cumulus masses, containing the ovulated oocytes, were released from the swollen ampullary region of the oviduct into sterile watchglasses containing M2 tissue culture medium. The cumulus masses were transferred via a Pasteur pipette to a watchglass containing about 1 ml of a freshly prepared 7% solution of Analar quality ethanol (BDH Chemicals Ltd.) in Dulbecco's phosphate buffered saline (PBS). They were retained in this solution for 7 mins at 37°C on a hotplate (Kaufman, 1982). The cumulus masses were then washed through two changes of M2 and one of M16 tissue culture medium in watchglasses. They were then transferred to plastic tissue culture dishes containing drops of M16 under paraffin oil and incubated for 5-6 hours at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. At the end of this time, the adherent cumulus cells were removed with (1mg/ml) hyaluronidase and the various classes of parthenogenone induced separated into different groups.

Four classes of parthenogenone could be determined at this stage (see Figure 2.1), but only the single-pronuclear haploid and the two-pronuclear diploid parthenogenones were examined further. These classes of embryos were transferred separately to pseudopregnant recipients

and, at various times after the hCG injection, the embryos were recovered from either the oviducts or the uteri of the recipients. Air-dried preparations were made, the embryos stained and their cell number established.

## **2.23 THE MICROMANIPULATION OF PRONUCLEI TO PRODUCE TRIPLOID**

### **EMBRYOS**

The enucleation/injection and holding pipettes for the micromanipulatory procedures were made from borosilicate capillary tubing GC100T-15 (Clark Electromedical Instruments). The injection pipettes were pulled on a moving-coil microelectrode puller model 753 (Campden Instruments Ltd.) at settings that would provide a suitable size of taper at the end of the pipette. The holding pipettes were pulled by hand over a flame.

Both the enucleation/injection and holding pipettes were modified for use on a Research Instruments Ltd. microforge. The taper of the enucleation/injection pipette was cut at a diameter of 20 $\mu$ m and bevelled at an angle of 25<sup>0</sup> on a K.T.Brown Type micro-electrode beveler model BV-10 (Sutter Instrument Company). The bevelled end was then gently heated and pulled to a sharp tip. The holding pipette was cut at a diameter of 100 $\mu$ m. The cut end was heated and polished until it had an internal diameter of 20 $\mu$ m. Both types of pipettes were bent in a manner to allow for positioning in the injection chamber. The enucleation/injection pipettes were washed in distilled water and Tween 80 (Sigma) and left to dry for a day.

The injection chamber consisted of a glass slide, siliconized with Sigmacote (Sigma), and a hollow plastic circular disc with slightly raised walls which was vaselined onto it. A large flat drop of medium was placed on the slide within the walls of the disc and covered with light paraffin oil.

The micromanipulatory procedures were carried out with Leitz micromanipulators and a Leitz Diavert inverted microscope with Nomarski interference contrast optics. The micromanipulatory procedures were carried out at a magnification of 500x (40x objective and



12.5x eyepieces), and a lower magnification was used for moving the embryos about in the injection chamber. The enucleation/injection pipette, and its associated tubing (ESCO Rubber Ltd.), were attached to a Hamilton microliter syringe (100 $\mu$ l) and all of these were filled with Fluorinert FC77 (Sigma). The holding pipette, and its associated tubing, were attached to a 2ml glass syringe and all of these were filled with paraffin oil. Each syringe was supported on a microliter syringe clamp (Research Instruments). Care was taken to exclude all air bubbles from the system.

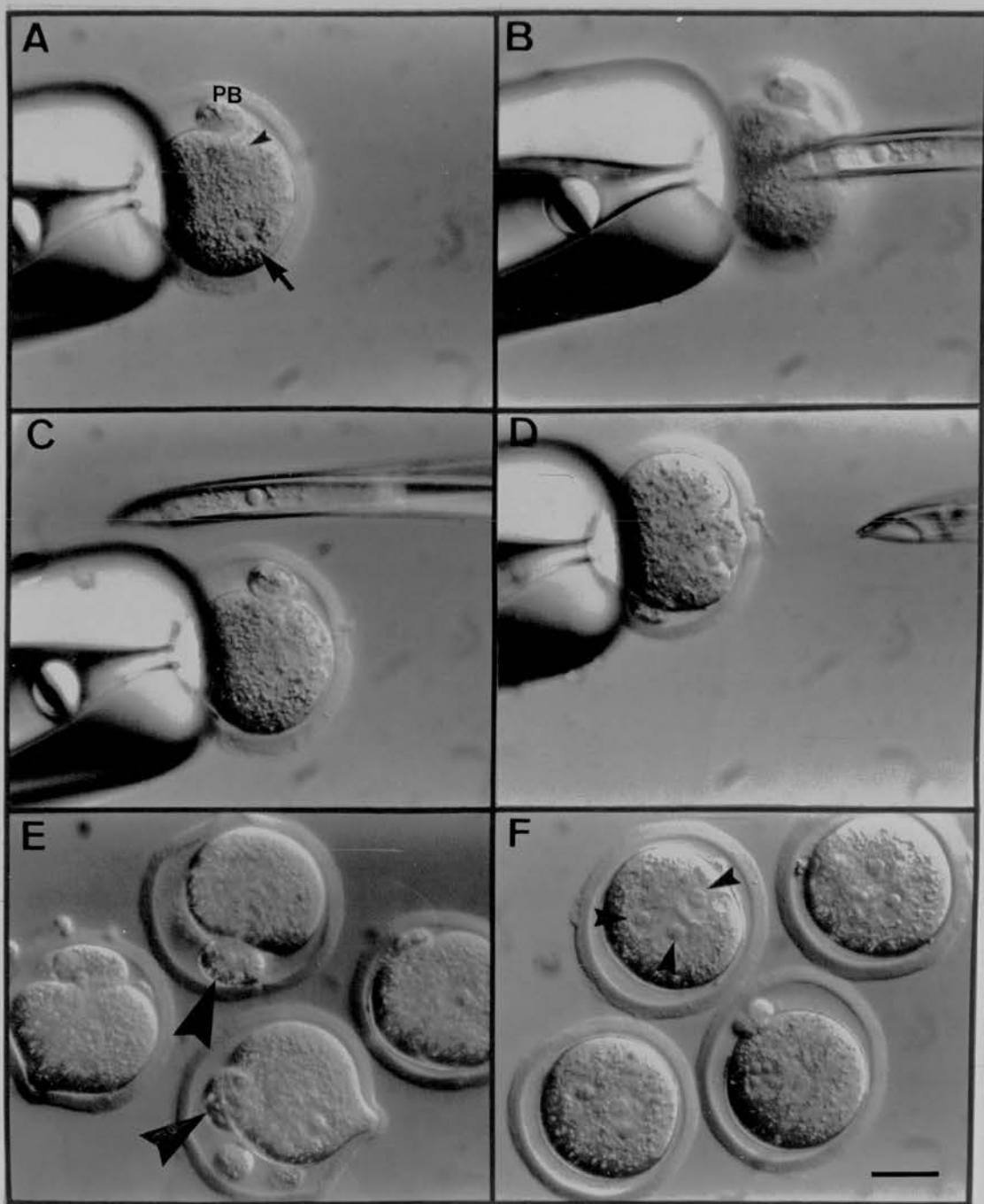
Following PMSG and hCG injections administered at 1500h, early pronucleate-stage fertilized embryos were isolated at about 10 a.m. in the morning on the day of finding a vaginal plug (hCG + 19 hours) and their cumulus cells were removed with hyaluronidase. In these early fertilized embryos, the female pronucleus is small and always located in close proximity to the second polar body, while the male pronucleus is much larger and located elsewhere in the cytoplasm, but usually in the subcortical zone located at the periphery of the egg. Embryos where it was impossible to distinguish between the two pronuclei were only used as recipient embryos for the injected pronuclei in the triploid studies.

The fertilized embryos were incubated for 45 minutes in M2 medium supplemented with 1 $\mu$ g/ml of cytochalasin D (Sigma) and 1 $\mu$ g/ml of colcemid (Sigma) prior to microinjection. Male or female pronuclei (depending on whether a diandric or digynic triploid was to be made) were isolated with a small volume of cytoplasm from "donor" embryos and inserted into the perivitelline space of a "recipient" 1-cell stage fertilized embryo using standard micromanipulatory techniques (McGrath and Solter, 1983). Batches of these fertilized embryos, with injected donor pronuclei, were washed through drops of M16 medium and then incubated for 1 hour in M16 at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. The injected pronuclei were then fused to the fertilized embryo cytoplasm by electrofusion. Micrographs describing these micromanipulatory procedures are shown in Figure 2.2.



Figure 2.2

- A) A recently fertilized diploid embryo at the pronuclear stage of development. The embryo, which is being held by a holding pipette, shows two obvious pronuclei. The larger one (arrow) is the male pronucleus and this is usually found in recently fertilized embryos in the subcortical zone at the periphery of the egg. The female pronucleus is smaller (small arrow head), and in recently fertilized embryos is always located in close proximity to the second polar body (PB).
- B) In this example, the male pronucleus is being withdrawn into the enucleation/injection pipette within a membrane-bound karyoplast from this "donor" embryo.
- C) Another fertilized embryo, the "recipient", the zona pellucida of which is to be penetrated by the enucleation/injection pipette containing the "donor" male pronucleus.
- D and E) The karyoplast containing the male pronucleus is injected into the perivitelline space of the "recipient" embryo (large arrow heads).
- F) Electrofusion of the karyoplast to the cytoplasm of the "recipient" embryo results, in this case, in diandric triploid embryos which show three clear pronuclei (medium-sized arrow heads). In the latter embryo, each of the pronuclei has a single nucleolus, though in the egg to the bottom right of the micrograph, one of the pronuclei has four obvious nucleoli.
- (scale bar = 30 $\mu$ m)



## 2.24 THE ELECTROFUSION OF PRONUCLEI TO RECIPIENT EMBRYOS TO FORM TRIPLOID EMBRYOS

The manipulated embryos to be fused were transferred into a low-electrolyte solution and a similar solution was also present in the fusion chamber. The fusion chamber and high voltage stimulator were identical to the one used for the electroactivation of cumulus-free oocytes. The high voltage pulse stimulator was set at 100 V and 130mAmps with a pulse duration of 50µsec. The manipulated embryos were individually placed between the wires to ensure that the injected pronucleus was orientated approximately parallel to the wires, and the pulse stimulator was triggered. The embryos were removed immediately and washed through 4 drops of M16 and then returned to the incubator. Fusion usually occurred within an hour but the non-fused embryos were exposed again to the pulse to induce fusion. These triploid trippronucleate embryos (either diandric or digynic) were then transferred unilaterally to the oviducts of recipients on the first day of pseudopregnancy.

## 2.25 THE ELECTROFUSION OF 2-CELL STAGE DIPLOID EMBRYOS TO FORM TETRAPLOID EMBRYOS

Female F1 hybrid mice were superovulated with PMSG and hCG, both administered at 1600h, and individually mated with stud males and the presence of a vaginal plug the next morning was taken as evidence of mating. Early in the morning on the second day of gestation, 2-cell stage embryos were recovered by flushing of the oviducts of the mated female mice. These embryos were incubated in equilibrated microdrops of M16 culture medium under paraffin oil in an atmosphere of 5% CO<sub>2</sub> in air at 37°C. The 2-cell stage embryos, whose blastomeres were to be fused, were transferred into a low-electrolyte solution and a similar solution was also present in the fusion chamber. The fusion chamber and high voltage stimulator were identical to the one used in the electroactivation of cumulus-free oocytes. The high voltage stimulator was set at 200V and 130mAmps with a pulse duration of 50µsecs. The embryos, in batches of 10, were placed between the two platinum wires of the fusion chamber and the pulse stimulator was triggered. The embryos were removed immediately and washed through four drops of M16 culture medium and were then returned to the incubator. Within 15-30 minutes, the two blastomeres had fused in a very high proportion of cases to form a single blastomere. Embryos

whose blastomeres had not fused were again exposed to the electrical pulse to initiate fusion. The 1-cell stage tetraploid embryos were then transferred unilaterally to the oviducts of recipient mice on the first day of pseudopregnancy.

## **2.26 SURGICAL TRANSFER OF EMBRYOS TO THE OVIDUCTS OF PSEUDOPREGNANT RECIPIENTS**

A small proportion of F1 hybrid male mice were vasectomised and their sterility was proven with matings to F1 hybrid female mice which subsequently did not become pregnant. Spontaneously cycling F1 hybrid female mice (of approximately 20 grams in weight) were caged the evening before the surgical transfer procedures with these males, and at least a quarter of these females mated. Vaginal plugs were checked the next day as evidence of mating and this was considered to be the first day of pseudopregnancy. Embryos were always transferred to the oviducts of female mice at this stage of pseudopregnancy.

The recipient mice were weighed and anaesthetised with a freshly prepared solution of Avertin (Winthrop). Avertin is composed of tribromomethyl alcohol in tertiary amyl alcohol and this stock solution was stored for several months in the dark. A 1.2% solution of Avertin was dissolved in warm 0.9% saline and the solution was mixed vigorously. One intraperitoneal injection of 0.02ml Avertin per gram of body weight anaesthetised the mice almost immediately for approximately 30-60 minutes. One mouse was anaesthetised at a time.

A mouth-controlled pipette was then loaded with the embryos to be transferred. The embryos were transferred in M2 culture medium in batches of between 8-12 (where possible). The anaesthetised mouse was laid on its abdomen on absorbent paper and its back was wetted with alcohol to keep its hairs out of the incision. A right dorsolateral incision, of about 1cm in length, was made in the mid-lumber region to expose the peritoneum which was then cut. The ovary, and its attached fat pad, were located on the back wall of the peritoneal cavity and they were gently pulled out of the mouse bringing the oviduct and uterus with them. The fat pad usually adhered to the skin of the mouse and this prevented the oviduct from sliding back into

the mouse. The mouse was then transferred to the dissecting microscope and the open end of the oviduct, the infundibulum, was located. A fine pair of watchmaker's forceps held the infundibulum up and out of the coils of oviduct and the fine mouth-controlled pipette, containing the embryos to be transferred, was inserted into it. The embryos were then gently expelled into the oviduct. The oviduct, ovary and fat pad were then re-inserted into the peritoneal cavity and the peritoneum was sewn up with a single stitch. The skin of the mouse was clipped together with surgical wound clips and the mouse was ear-tagged and left to recover from the anaesthetic in a warm cage. The transferred embryos were now considered to be in their first day of gestation.

## **2.27 MORPHOMETRIC ANALYSIS**

Diandric and digynic triploid embryos were recovered between 9-12 days of gestation and for tetraploid embryos this was 10-16 days of gestation. Control diploid embryos were recovered at comparable stages of gestation. These embryos were fixed and embedded for wax histology. Serial sections of the embryos were made at a nominal thickness of 7 $\mu$ m, or 8 $\mu$ m for larger embryos, and stained with haematoxylin and eosin.

### **2.27.1 ANALYSIS OF THE STAGE OF POSTIMPLANTATION DEVELOPMENT**

The exact developmental age of the embryos was established by comparing their cranial/postcranial morphological/histological features with those of diploid control embryos in Professor Kaufman's reference collection of serially-sectioned mouse embryos, based on the morphological staging schemes of Theiler (1989) and Kaufman (1990, 1992b). In these schemes, somite number, limb bud development, and the degree of differentiation of a wide variety of developing organ systems are compared in order to provide as accurate an assessment as possible of the developmental (rather than chronological) age of the embryos.



### 2.27.2 ESTIMATING CELLULAR AND NUCLEAR VOLUME FROM SERIAL SECTIONS

An estimation of cellular or nuclear volume, of the cells under study, were obtained from measurements of their diameter using a Kontron/MOP Videoplan image analysing computer linked to a Leitz Laborlux 12 microscope via a Hitachi type 3000 colour television camera. The program automatically calculates the volume from the diameter of the cell or nucleus.

Measurements were made on sections viewed using an oil immersion objective (x100). All cells and nuclei in the selected fields were measured on sections at not less than 20 $\mu$ m intervals along the longitudinal axes of the embryos in order to avoid analysing individual cells on more than one occasion. 100 measurements were made (where possible) on the cells studied in each embryo.

The rationale behind this estimation of volume is based on Abercrombie (1946). If it is assumed that all nuclei (or cells) within a tissue have a constant spherical or near- spherical shape and an average diameter of less than that of the section thickness, then they will be wholly present in the majority of sections studied. Hence, over 100 measurements, nuclei or cells with a diameter of less than 7 $\mu$ m (or 8 $\mu$ m) will give an average unbiased estimate of volume (cellular or nuclear) for that embryo. By the same argument, it follows that if the cells have a diameter of greater than the section thickness, an underestimate of cellular or nuclear volume may result. Because of the size of the cells studied (see later), the results will show an underestimate of the true cellular or nuclear volume of that embryo. In the case of the nucleated red blood cells, cells were only analysed if the nucleus was also present in the section analysed.



## 2.28. STATISTICAL ANALYSIS

### 2.28.1. MEAN

The sample mean,  $\bar{x}$ , is the arithmetic average obtained by dividing the sum of all the scores,  $\Sigma x$ , by the number of scores,  $n$ . It can be defined as follows:

$$\bar{x} = \frac{\Sigma x}{n}$$

The sample mean is an unbiased estimator of the population mean.

A mean on its own is meaningless unless it is accompanied by a measure of dispersion. One such measure is the standard deviation.

### 2.28.2. STANDARD DEVIATION

The standard deviation is a measure of the differences between each observation and the mean value, it is equal to the square root of the variance. An unbiased estimate of the standard deviation ( $s$ ) of a population is,

$$s = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

### 2.28.3. STANDARD ERROR OF THE MEAN

The SEM for one sample is an estimate of the standard deviation that would be obtained from the means of a large number of samples drawn from the parent population. The SEM of a sample can be calculated by dividing the standard deviation of the sample by the square root of the sample size. So,

$$\begin{aligned} \text{SEM} &= \frac{\text{standard deviation}}{\text{square root of sample size}} \\ &= \frac{s}{\sqrt{n}} \end{aligned}$$

#### 2.28.4. $\chi^2$ TEST

This test can be used to determine whether an observed frequency distribution departs significantly from a hypothesised frequency distribution. In my case, it was used to test whether proportions differed between samples. The formula for this test is given by:

$$\chi^2 = \left\{ \frac{(f_O - f_E)^2}{f_E} \right\}$$

where  $f_O$  is the observed frequency and  $f_E$  is the expected frequency.

When there were only two proportions a  $2 \times 2$  contingency table was used with the degrees of freedom equal to 1. The Fisher exact probability test was used when  $f_E$  was less than 5, the significance level was set at  $p = 0.05$ .

#### 2.28.5. ANALYSIS OF CELL DOUBLING TIMES

A logarithmic relationship was found to exist between the number of cells in an embryo and time. Least squares methods were used to fit regression lines relating the logarithm of the number of cells to time and the regression was weighted by the sample size for each mean value. The resulting gradients were used to estimate the time period over which the number of cells doubled. If  $N_t$  and  $N_0$  represent the number of cells at time  $t$  and time 0 respectively, and  $b$  represents the gradient of the regression line, then,

$$\begin{aligned} \ln(N_t) &= \ln(N_0) + bt \\ N_t &= N_0 e^{bt} \end{aligned} \quad (1)$$

Now for two arbitrary times  $t_1$  and  $t_2$ ,

$$\begin{aligned} \frac{N_{t_2}}{N_{t_1}} &= \frac{e^{bt_2}}{e^{bt_1}} && \text{from (1)} \\ &= e^{b(t_2 - t_1)} && (2) \end{aligned}$$

If  $N_{t_2} = 2N_{t_1}$  then from (2) we get,

$$\text{cell doubling time} = t_2 - t_1 = \frac{1}{b} \ln \left( \frac{N_{t_2}}{N_{t_1}} \right) = \frac{\ln 2}{b}$$

From the standard error of the gradient, the standard error of the doubling time was calculated. The gradients of the regression lines were used to test for differences in cell doubling times.

#### 2.28.6. TESTING FOR DIFFERENCES BETWEEN REGRESSION LINES

To test whether there was a significant difference between two fitted regression lines, a combined line using all the data was fitted. Fisher's F test was used to determine whether the variation about this single combined line was significantly higher than the pooled variance of the original two lines. If this was the case, then the two original regression lines were assumed to be different.

### CHAPTER 3

#### THE CLEAVAGE RATES OF HAPLOID AND DIPLOID PARTHENOGENETIC MOUSE EMBRYOS DURING THE PREIMPLANTATION PERIOD

### 3.1 INTRODUCTION

Parthenogenetic embryos provide a useful means of studying a parental influence on development, as well as the possible effect of ploidy, without the technical difficulties involved in nuclear manipulations.

While some postimplantation development has been achieved in both diploid (Kaufman et al., 1977) and haploid parthenogenetic embryos (Kaufman, 1978), it would appear that successful completion of development needs both a maternal and paternal genetic component (McGrath and Solter, 1984; Mann and Lovell-Badge, 1984; Surani et al., 1984; Barton et al., 1984). However, blastocyst formation can be readily achieved in diploid gynogenones and androgenones, though the former type of embryos consistently develop better than the latter type embryos (Markert and Petters, 1977; Modliński, 1980; Borsuk, 1982; Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984, 1986; Kaufman et al., 1989a; Latham and Solter, 1991; Mann and Stewart, 1991; Kono et al., 1993). Blastocyst formation can also be achieved in haploid and diploid parthenogenones (Witkowska, 1973a; Kaufman and Sachs, 1976; Kaufman, 1981, 1983). While it appears, from a superficial analysis of parthenogenetic embryos, that during the preimplantation period no obvious detrimental effect of the absence of a paternal genome occurs, an analysis of cell number and cleavage rate may, however, provide a means of establishing whether this is indeed the case. Furthermore, this type of analysis of haploid and diploid parthenogenetic development may also reveal the effect that ploidy has on preimplantation development, without the need for micromanipulatory procedures.

Therefore, I decided to undertake a detailed evaluation of the cleavage rate *in vivo* of preimplantation haploid and diploid parthenogenones following their transfer to appropriate recipients. Witkowska (1973a,b) activated newly ovulated mouse eggs *in situ* with an electric shock, and examined the development of the parthenogenetically activated eggs on subsequent days of gestation. However, these studies were difficult to interpret, because a mixture of

haploid and diploid parthenogenetic embryos almost invariably resulted from this activation technique (Tarkowski et al., 1970). I believe that it is preferable to use *in vitro* activation techniques, as this allows the selective transfer of recently activated haploid or diploid parthenogenetic embryos to pseudopregnant recipients, and allows their individual development to be analysed and compared with that of other classes of parthenogenones, as well as with control diploid embryos studied under similar experimental conditions.



### 3.2 MATERIALS AND METHODS

8- to 12-week-old F1 hybrid female mice were superovulated and then autopsied at 19-19.5 hours after the HCG injection (PMSG and hCG administered at 1400h). The ovulated cumulus masses were transferred to a 7% solution of ethanol to activate the eggs, as described previously. After washing, the cumulus masses were cultured for 5-6 hours. At the end of this time, the adherent cumulus cells were removed with hyaluronidase, and the various classes of parthenogenone induced separated into different groups.

Four classes of parthenogenones could be determined at this stage, namely (a) oocytes which contained a single (haploid) pronucleus, having previously extruded a second polar body, (b) oocytes which contained two (haploid) pronuclei in the absence of second polar body extrusion, (c) oocytes which underwent "immediate cleavage" in which two equal-sized blastomeres had formed, each containing a single (haploid) pronucleus, one of the blastomeres representing the second polar body, and (d) oocytes in which a single (diploid) pronucleus developed in the absence of second polar body extrusion (see Figure 2.1). I was concerned in this study with the preimplantation development of groups (a) and (b). Between 10-12 embryos belonging to either group (a) or (b) were then transferred unilaterally to the oviducts of recipients on the afternoon of the first day of pseudopregnancy.

In the control series, F1 hybrid female mice were superovulated with PMSG and hCG at 1500h and mated individually with fertile homozygous Rb(1.3)1Bnr males. Pronucleate-stage eggs were isolated and cultured for 5-6 hours in M16 and then transferred unilaterally to recipients.

At various time points between 48-105h after hCG, the recipient mice were killed and the transferred embryos recovered. In all instances, note was taken of the gross morphological appearance of individual embryos, and from which location within the reproductive tract they

were recovered. Air-dried preparations were made of these embryos using the technique described by Tarkowski (1966), and the spreads were then stained to enable accurate cell counts to be made.

### 3.3 RESULTS

The findings from this study are shown in Table 3.1 and Figures 3.1 and 3.3. The data can be considered firstly in relation to total cell count for the three groups and secondly in relation to the location and morphological appearance of the embryos.

#### 3.3.1 CELL DOUBLING TIME FOR FERTILIZED DIPLOID CONTROL EMBRYOS AND *IN VITRO* ACTIVATED TWO-PRONUCLEAR DIPLOID AND SINGLE-PRONUCLEAR HAPLOID PARTHENOGENETIC EMBRYOS AFTER ISOLATION AT THE 1-CELL STAGE AND SUBSEQUENT TRANSFER TO PSEUDOPREGNANT RECIPIENTS.

Directly after the embryos from these groups had been isolated from the pseudopregnant recipients at specific times after the hCG injection for inducing their ovulation, their cell number was established. The values obtained were converted to natural logarithms, and were plotted graphically against time after hCG injection (see Figure 3.1). Regression lines were drawn for each class of embryo, and this enabled their individual cell doubling time to be calculated. For the diploid control group, the cell doubling time between 48-105h after the HCG injection was found to be  $12.74 \pm 1.17\text{h}$ .

The cell doubling time for the two-pronuclear diploid parthenogenetic group between 50-99h after the hCG injection was found to be  $12.25 \pm 0.34\text{h}$ . This was not statistically different from the value obtained for the control series ( $p > 0.05$ ).

The cell doubling time for the single-pronuclear haploid parthenogenetic group between 50-104h after the hCG injection was  $15.25 \pm 0.99\text{h}$ . The gradient of this line was statistically different from the gradient of the diploid parthenogenetic embryos ( $p < 0.05$ ). Representative air-dried preparations of parthenogenetic haploid and diploid embryos are shown in Figure 3.2.

Table 3.1 The preimplantation development of control diploid mouse embryos and parthenogenetic diploid and haploid mouse embryos.

| Group                         | Time of isolation<br>(hours post hCG) | No.embryos transferred | No.embryos recovered<br>(total) healthy (%)healthy | No.analysed to establish cell number | Mean cell number<br>(±SEM) |
|-------------------------------|---------------------------------------|------------------------|--|--------------------------------------|----------------------------|
| (i) Control diploids          | 48                                    | 58                     | (68)* 61* (89.7)                                   | 58                                   | 2.22 (± 0.08)              |
|                               | 75                                    | 55                     | (62)* 51 (82.2)                                    | 46                                   | 6.46 (± 0.51)              |
|                               | 80                                    | 70                     | (83)* 56 (67.5)                                    | 50                                   | 13.74 (± 0.67)             |
|                               | 96                                    | 65                     | (65) 53 (81.5)                                     | 41                                   | 30.51 (± 2.44)             |
|                               | 105                                   | 106                    | (83) 57 (68.7)                                     | 41                                   | 51.90 (± 3.32)             |
| (ii) Diploid parthenogenones  | 50                                    | 46                     | (46) 46 (100)                                      | 46                                   | 2.30 (± 0.11)              |
|                               | 76                                    | 69                     | (75)* 49 (65.3)                                    | 48                                   | 9.79 (± 0.52)              |
|                               | 92                                    | 76                     | (57) 46 (80.7)                                     | 41                                   | 27.00 (± 1.54)             |
|                               | 99                                    | 130                    | (106) 73 (68.9)                                    | 61                                   | 35.20 (± 1.99)             |
|                               |                                       |                        |  |                                      |                            |
| (iii) Haploid parthenogenones | 50                                    | 60                     | (68)* 51 (75.0)                                    | 50                                   | 2.10 (± 0.06)              |
|                               | 76                                    | 123                    | (105) 82 (78.1)                                    | 64                                   | 8.53 (± 0.38)              |
|                               | 93                                    | 91                     | (95)* 64 (67.4)                                    | 55                                   | 16.58 (± 0.79)             |
|                               | 99                                    | 128                    | (115) 91 (79.1)                                    | 73                                   | 16.62 (± 1.07)             |
|                               | 104                                   | 53                     | (44) 32 (72.7)                                     | 21                                   | 26.09 (± 4.14)             |

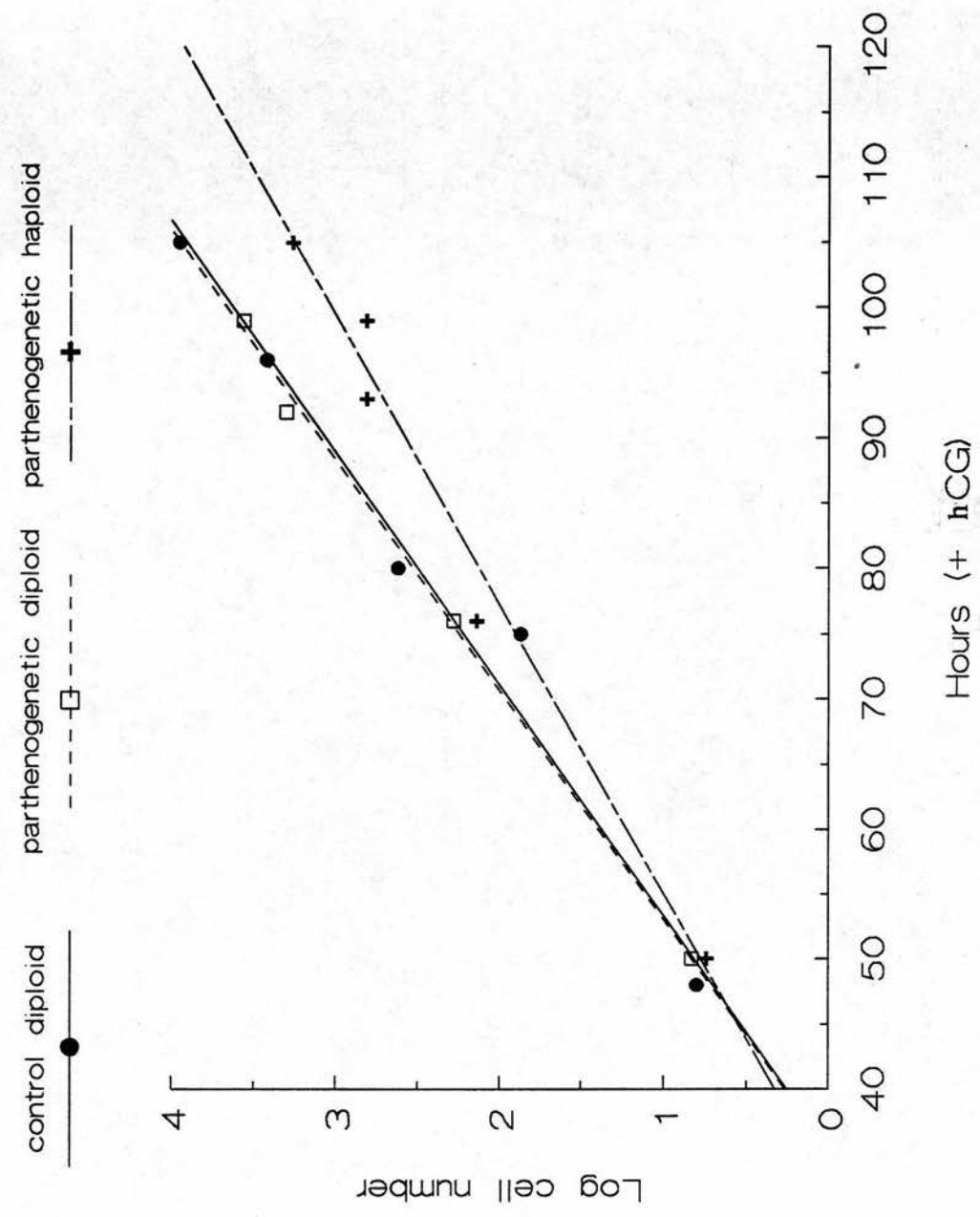
\* It is assumed that these embryos must have resulted from the parthenogenetic activation of the recipients' eggs, due to the activating stimulus of the anaesthetic employed during the transfer procedure (Kaufman, 1975; Kaufman and Sachs, 1975).

The overall % of mice becoming pseudopregnant after surgical transfer of control 1-cells, diploid parthenogenones and haploid parthenogenones was 77.8%, 87.8% and 89.6% respectively.

Figure 3.1

Log total cell number of embryos isolated at various times during the preimplantation period, plotted against time after hCG injection to induce ovulation in (i) control diploid embryos (ii) diploid parthenogenetic embryos, and (iii) haploid parthenogenetic embryos. By regressing the log cell number against time, it was possible to estimate the gradient of each line using least squares. The regression was weighted using the sample size for each mean value. From the gradient, it was possible to estimate the average cell doubling time for each series. No significant difference was found to exist between the cell doubling times of diploid parthenogenones and the control diploid embryos, but a significant difference was found to exist between the cell doubling times of the diploid parthenogenones and the haploid parthenogenones (see text for further details).

Figure 3.1





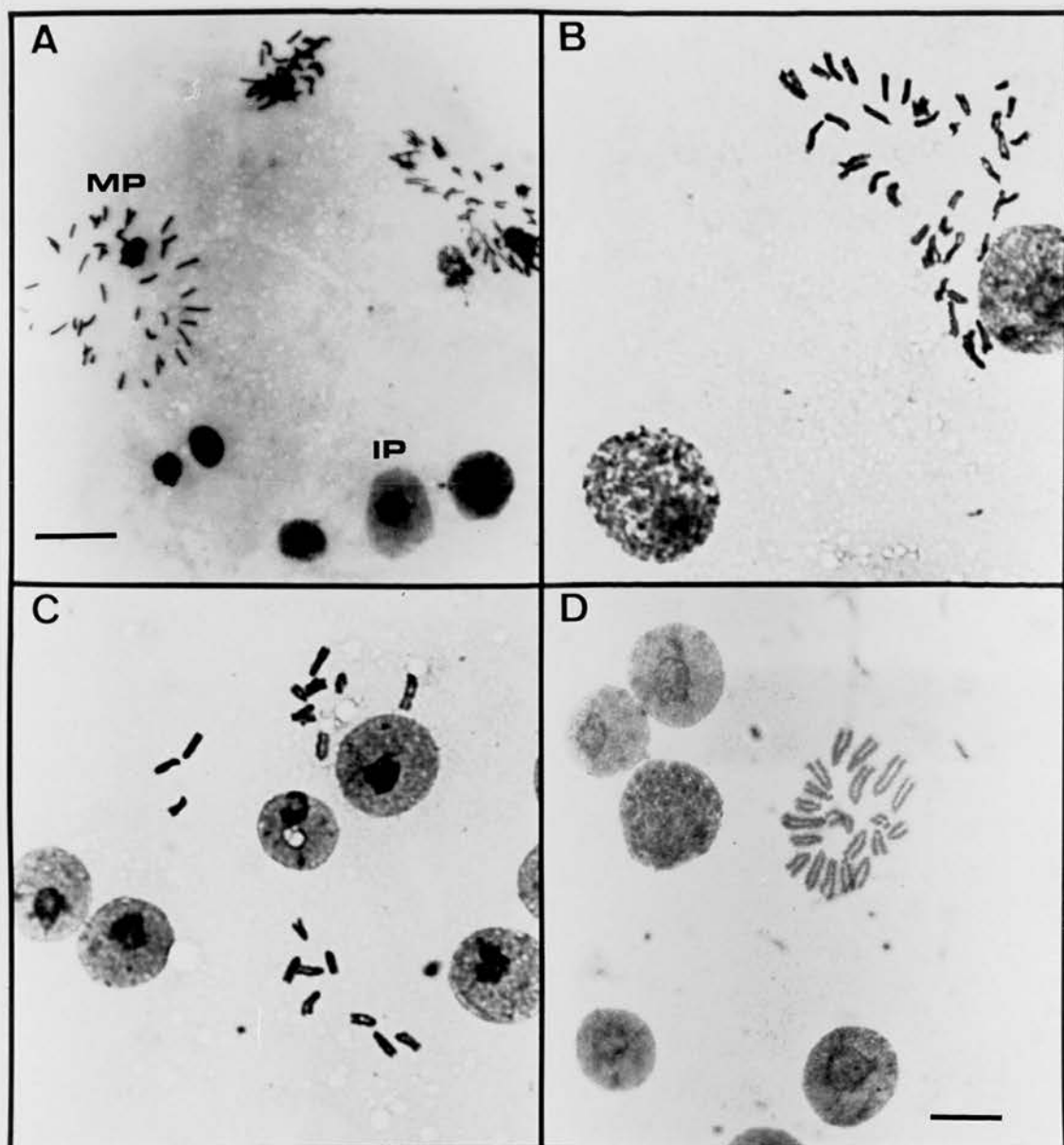
**Figure 3.2**

Representative air-dried preparations of early preimplantation parthenogenetic mouse embryos. Activated oocytes were transferred to pseudopregnant recipients and recovered at various time points after the original hCG injection to induce ovulation. (scale bar = 30µm)

A) Early preimplantation diploid parthenogenone. Each metaphase plate (MP) shows 40 chromosomes. Some cells are clearly at other stages of mitotic division. Interphase nuclei (IP) are also present.

B) High power view of a diploid metaphase plate showing 40 chromosomes.

C and D) Haploid parthenogenones with metaphase plates showing 20 chromosomes in each.  
(B, C, D: scale bar = 15µm)



3.3.2. THE LOCATION AND MORPHOLOGICAL APPEARANCE OF CONTROL AND PARTHENOGENETIC EMBRYOS AT VARIOUS TIMES AFTER THEIR TRANSFER TO PSEUDOPREGNANT RECIPIENTS.

(i) FERTILIZED DIPLOID CONTROL EMBRYOS

All of the healthy embryos recovered from the diploid control group at 80h after the original hCG injection were compacted morulae or in a few cases precompacted embryos with a mean cell number ( $\pm$  S.E.M.) of  $13.74 \pm 0.67$ . They were exclusively located in the oviduct. By 96h, nearly 23% of all the healthy embryos were blastocysts, and the rest were morulae or in a few cases precompacted embryos. Embryos could be found in both the oviduct and the uterus at this time. By 105h, all the embryos were located in the uterus and nearly 37% of these were zona-free blastocysts, 29% were expanded zona-intact blastocysts, and the remaining embryos (34%) were morulae or (rarely) precompacted embryos.

(ii) DIPLOID PARTHENOGENETIC EMBRYOS

The diploid parthenogenones followed a similar progress to (i) above.

At 76h after the original hCG injection, all the healthy embryos recovered were morulae (mean cell number  $\pm$  S.E.M.:  $9.79 \pm 0.52$ ), and were all located in the oviduct. By 92h, morulae and the first blastocysts were observed, and they comprised 15% of all the healthy embryos recovered at this time. Embryos could be found at this time in both the oviduct and the uterus. By 99h, zona-free blastocysts were recovered from the uterus, though the majority of embryos were zona-intact blastocysts and morulae, and were recovered from both the oviduct and the uterus.

(iii) HAPLOID PARTHENOGENETIC EMBRYOS

In the case of the haploid parthenogenones, in the period of time studied, embryos had progressed only to the morula stage, (and rarely to the blastocyst stage (approximately 5%) ) or were precompacted embryos. Curiously, by 76h after the original hCG injection, embryos

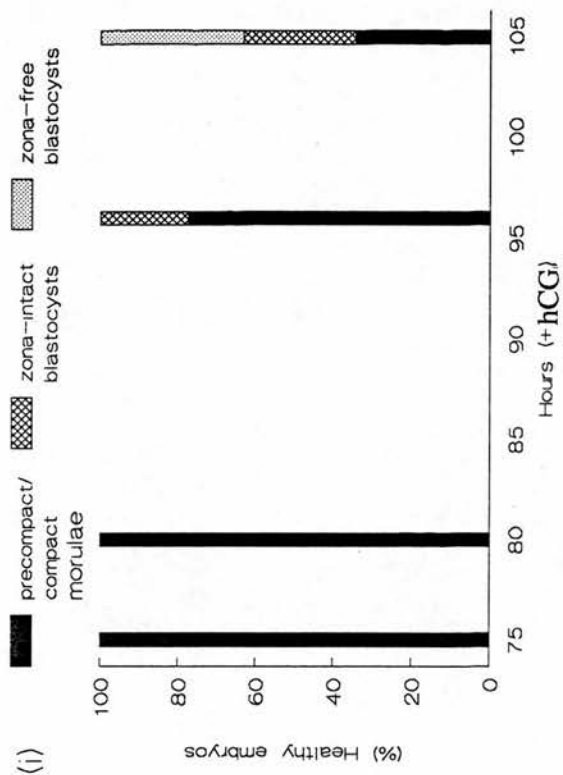
were recovered from both the oviduct and the uterus, and had progressed further in the female tract than the embryos of groups (i) and (ii), at approximately the same time after the hCG injection.

The morphological appearance, whether precompacted embryos and/or morulae, zona-intact or zona-free blastocysts, of embryos from groups (i) - (iii), above, at various times after the hCG injection to induce ovulation, are illustrated in Figure 3.3.

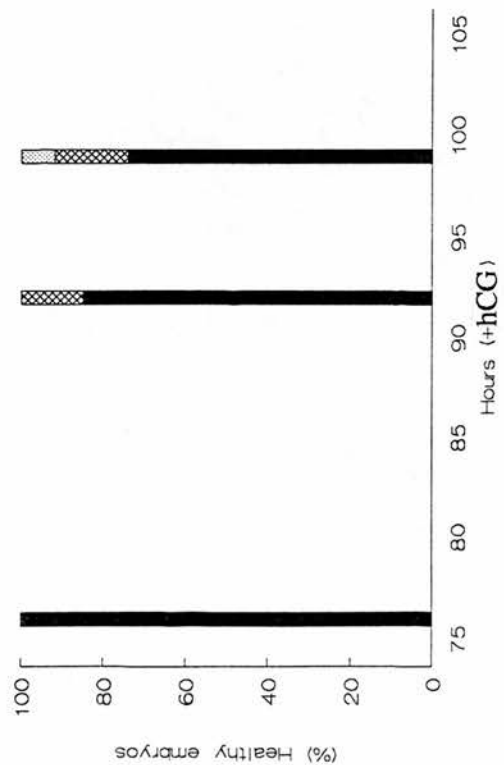
**Figure 3.3**

Morphological appearance of embryos isolated at various times during the preimplantation period, plotted against time after hCG injection to induce ovulation in (i) control diploid embryos, (ii) diploid parthenogenetic embryos, and (iii) haploid parthenogenetic embryos. (morphological appearance based on number of recovered healthy embryos from Table 3.1)

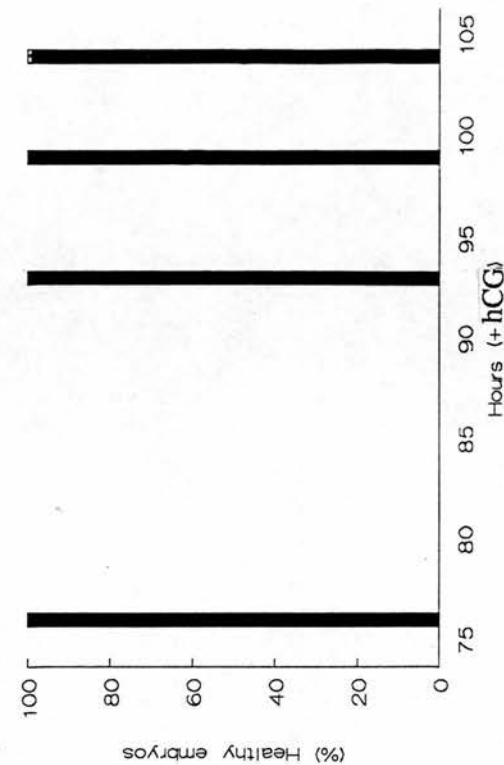
Figure 3.3



(ii)



(iii)





### 3.4 DISCUSSION

I have found that the parthenogenetically activated single-pronuclear (uniform) haploid mouse embryos had a slower cleavage rate during the preimplantation period than that of heterozygous diploid parthenogenetic embryos and fertilized control diploid embryos when all three groups were transferred to pseudopregnant recipients. The diploid parthenogenones, however, cleaved at approximately the same rate as the fertilized control diploid embryos. This result suggests that while the lack of one genetic component (in the case of the haploid parthenogenones) increases the duration of the cell cycle and consequently slows down their development, the presence of two maternally-derived genetic components does not appear to do so. Indeed, at least during the preimplantation period, the presence of such an abnormal diploid genome appears to have little obvious detrimental effect in terms of cleavage rate and the ability of these embryos to form apparently morphologically normal blastocysts. In addition, I recovered about 70% healthy haploid and diploid parthenogenetic embryos and about 80% of the control diploids after their transfer to recipients. This suggests that a reduced cell number, as found in the haploids, does not limit the viability of such embryos during the period up to implantation.

These results confirm what others have found. Thus, haploid parthenogenones have been reported to cleave slower than diploid parthenogenones in tissue culture (Kaufman and Sachs, 1976; Kaufman, 1981; Tsunoda and Shioda, 1988). Furthermore, Kaufman and Sachs (1976) reported that the immediate cleavage and the delayed cleavage type parthenogenones develop better than the single-pronuclear uniform haploids. It is of interest that the duration of the first cleavage mitosis is almost identical in diploid parthenogenetic and fertilized embryos, while the duration of this mitosis in haploid parthenogenones is significantly slower (Kaufman, 1973b). If a similar pattern also occurs in subsequent divisions, this might provide a partial explanation for the present findings, though it should be noted that in that study both the haploid and diploid parthenogenones entered the first cleavage mitosis at approximately the same time after activation.

In contrast to the study of Witkowska (1973a), who reported that the parthenogenetic haploid embryos on day 5 in her study had more cells than the diploid parthenogenones, and that the ability of embryos to reach the blastocyst stage was not connected to ploidy, less haploid parthenogenetic embryos in this study reached the blastocyst stage in comparison to diploid parthenogenones and fertilized diploid controls. Opas (1977) recovered only 4 haploid morulae on the 4th, and 5th day from the 22 haploid embryos that were transferred at the pronuclear stage. The haploid embryos analysed by Edwards (1958b) apparently had fewer cells than were present in control diploid embryos. This finding, however, he attributed to a delay in the onset of the first cleavage division, rather than being a consequence of their having a slower cleavage rate. Haploid gynogenetic and androgenetic mouse embryos produced by microsurgically removing one pronucleus from a fertilized embryo, showed a markedly delayed rate of development during the preimplantation period when compared with that of "homozygous" diploid embryos produced by the diploidisation of similar eggs following their exposure to cytochalasin B (Modliński, 1975; 1980; Markert and Petters, 1977; Surani and Barton, 1983; Surani et al., 1986). Most of the haploid embryos in these studies remained at the morula stage, and failed to progress to the blastocyst stage, whereas the homozygous diploid embryos regularly formed blastocysts. Gynogenetic diploids produced by suppressing the 2nd polar body and subsequently removing the male pronucleus can reach the blastocyst stage (Borsuk, 1982; Surani et al., 1984), and a smaller proportion of heterozygous androgenetic diploids can (Barton et al., 1984; McGrath and Solter, 1986; Latham and Solter, 1991; Mann and Stewart, 1991; Kono et al., 1993). Modliński (1975) noted though, that only the haploid eggs in which the male pronucleus was removed (i.e. the gynogenones) survived to the fifth day in culture. One possible explanation was proposed for this finding, namely that damage was more likely to have been sustained by the cytoskeletal elements and vitelline membrane during the removal of the female (but not the male) pronucleus, and that this may have had a detrimental effect on the viability of the androgenones. In addition, since about 25% of diploid androgenetic preimplantation embryos possess a YY sex chromosome constitution, they would not in any case be expected to reach the blastocyst stage (Burgoyne and Biggers, 1976). Despite such a poor reported preimplantation development, a proportion of heterozygous

diploid androgenones implant, and are capable of a limited degree of postimplantation development (Barton et al., 1984; Kaufman et al., 1989a). The lack of mitosis in those embryos examined by Witkowska (1973a) made it likely that she may have been dealing with haploid/diploid mosaic parthenogenones, rather than pure haploids. My experiment has been able to follow single-pronuclear uniform and two-pronuclear parthenogenones, produced after activation *in vitro* and subsequently transferred for development *in vivo*. This approach is theoretically superior to Tarkowski et al. (1970) and Witkowska (1973a, b) who activated ovulated eggs *in vivo* by electric pulse. They were unable to distinguish between the various classes of parthenogenones, although they were able to show that these embryos are capable of postimplantation development.

Kaufman and Sachs (1976) noted that under their culture conditions, and unlike the finding in this study, fertilized control diploid embryos cleaved slightly more rapidly than did their diploid parthenogenones. Since, it is well known that *in vitro* development is generally considered suboptimal when compared with *in vivo* development (see, for example Bowman and McLaren, 1970; Streffer et al., 1980; Harlow and Quinn, 1982; Bavister, 1988; Evsikov et al., 1990), it is possible that those embryos which may already be at a genetic disadvantage may be more sensitive to the environmental conditions *in vitro* than genetically normal embryos. I have overcome this theoretical disadvantage by transferring the embryos to recipients to enable development to proceed *in vivo*.

An explanation that may partly account for the slower cleavage rate of the single-pronuclear haploid compared to the diploid parthenogenones and the diploid control embryos relates to the suboptimal nuclear-cytoplasmic ratio of the haploid embryos. This is consistent with the findings of McGrath and Solter (1986) who compared the developmental ability of single-pronuclear haploid embryos, produced by the removal of a single male or female nucleus, with haploid embryos that underwent normalization of their nuclear-cytoplasmic ratio by removing a single pronucleus and additionally half the cytoplasmic volume of the zygote. They observed an increased proportion of the cytoplasm-depleted haploid embryos developed to the

morula/blastocyst stage. This finding was similar to the earlier observation that an increased proportion of parthenogenones that underwent either immediate cleavage or delayed cleavage (and thus normalized their nuclear-cytoplasmic ratio) developed beyond the 4- cell stage when compared to single-pronuclear haploid embryos that possessed a decreased nuclear-cytoplasmic ratio (Kaufman and Sachs, 1976; Kaufman, 1981). Similarly, others have observed that a greater proportion of haploid embryos produced by zygote bisection (Tarkowski and Rossant, 1976; Tarkowski, 1977) complete preimplantation development than haploids produced by pronuclear removal alone (Modliński, 1975, 1980).

The fact that less haploid than diploid parthenogenones reach the blastocyst stage (Kaufman, 1981, and this study) and that their cell number, even when they do so, is much reduced in comparison to diploid parthenogenones when maintained under similar conditions either *in vivo* or *in vitro*, may mean that they are less capable of evoking a decidual reaction and implanting in the uterus during the limited period of time that the uterus is in a receptive phase. In this study, while most of the control diploid embryos and the diploid parthenogenetic embryos were at the blastocyst stage at approximately 99 hours after the hCG injection, none of the haploid parthenogenetic embryos had achieved this stage by this time. Kaufman (1981) noted that some haploid parthenogenetic morulae that initially appeared to compact normally go through a "decompaction" phase before progressing to form apparently normal blastocysts. This "decompaction" phase appears to be a feature of some haploid embryos, and does not appear to occur in diploid parthenogenetic embryos nor in normal fertilized diploid embryos. Haploid embryos clearly have a reduced total number of cells at this time, and this inevitably reduces the number of cells likely to be located in their inner cell mass (ICM). By the time that implantation would normally occur, the total number of ICM cells present may be insufficient to allow the development of the embryo proper to occur, despite the presence of a relatively normal decidual response (Ansell and Snow, 1975; Snow et al., 1976). Haploid parthenogenones with few or no ICM cells have been observed previously, though it is believed that diploid parthenogenones almost always contain ICM cells (Kaufman, 1978). This observation might explain why haploid postimplantation development is so uncommon.

Immediate cleavage haploid parthenogenones can survive to the egg cylinder stage and contain healthy dividing cells, but proceed no further (Kaufman, 1978). The fact that immediate cleavage embryos have a normalized nuclear-cytoplasmic ratio and that they begin development with two blastomeres instead of one, may be advantageous for blastocyst cell number and their chances of implanting.

The potential that haploid and diploid parthenogenetic cells have for further development has also been examined in chimeric embryos. Diploid parthenogenetic mouse embryos are capable of surviving to the forelimb bud stage and have about 25 pairs of somites (Kaufman et al., 1977). Diploid parthenogenetic/diploid fertilized mouse chimeras, however, can reach birth. The parthenogenetic cells have been able to divide and differentiate and contribute to various tissues, although as development proceeds, these cells are actively selected against. Such chimeras have shown that diploid parthenogenetic cells can contribute to the brain, heart, germ cells, kidney and spleen, but a systematic elimination of parthenogenetic cells during embryonic development occurs in skeletal muscle, liver, pancreas and extra-embryonic membranes (Stevens et al., 1977; Surani et al., 1977; Stevens, 1978; Nagy et al., 1987, 1989; Fundele et al., 1989, 1990).

Single-pronuclear uniform haploid parthenogenetic/fertilized mouse chimeras can also develop to term, but only diploid parthenogenetic cells are found to be present in these newborns (Ito et al., 1991). The contribution of parthenogenetic cells to tissues, even in these chimerics, is very limited and the greatest contribution (30%) is found in the brain. This is indicative that a haploid chromosome constitution is unstable and a contribution to tissues only occurs if such cells are diploidised. Kaufman (1978) also found that the majority of presumptive parthenogenetic haploid egg cylinders in his study were haploid/diploid mosaics. It appears that haploid cells have an intrinsic difficulty with growth and differentiation in the postimplantation period, and that a diploid genome is more compatible with development.



Full development to term of diploid parthenogenones, gynogenones or androgenones has not been reported. This is believed to be a consequence of the absence of an opposite parental genome in these embryos, since both sets of parental genomes are believed to have different, but complementary roles, essential for successful development (Surani and Barton, 1983; Barton et al., 1984; McGrath and Solter, 1984; Mann and Lovell-Badge, 1984; Surani et al., 1984).

The fact that diploid parthenogenones develop well into the postimplantation period is compatible with results from molecular studies on such embryos. They have shown that the presence of two maternally-derived genomes has no detrimental effect on the way some synthesised gene products appear to be regulated. For example, it has been reported that the loss of oocyte-coded glucose phosphate isomerase (GPI) and the activation of the embryonic genome for GPI appears to be equivalent in homozygous diploid parthenogenones and fertilized embryos (Petzoldt, 1991), and activation of the embryonic genome in parthenogenetic and gynogenetic haploids occurs at the same time as in fertilized embryos (Surani et al., 1986). Therefore, gene regulation in such embryos is apparently able to occur even in the absence of a paternal genome.

A relatively high incidence of aneuploidy results from the use of ethanol activation. Kaufman (1982) found that approximately 19% of single-pronuclear uniform haploid parthenogenones were aneuploid. Furthermore, the incidence of aneuploidy has been found to increase with the duration of the exposure of the eggs to this activation stimulus (O'Neill and Kaufman, 1989). It must, therefore, be assumed that a proportion of the haploid parthenogenones in this study were aneuploid also, although I did not detect this. The development of aneuploid parthenogenetic embryos has been studied by Kaufman and Sachs (1975). These authors transferred recently activated haploid parthenogenetic embryos to recipients and recovered the embryos three days later. Cell counts revealed that the presence of an additional chromosome in some parthenogenones "did not hinder the early haploid embryonic development". However, haploid parthenogenones which had only 19 chromosomes were absent at this time



and it was thought that they probably failed to develop beyond the 2-cell stage. The influence of aneuploidy on preimplantation development has been reviewed by Dyban and Baranov (1987). It was reported that trisomy for at least 11 autosomes in the mouse has no detrimental effect on cleavage or blastulation. Monosomy appears to be more detrimental to development although Kaufman and Bain (1984) reported equal viability of monosomic and trisomic embryos up to the morula stage. The possible influence that aneuploidy might have on development is important to note and it might be instructive to repeat this experiment using a similar effective activation stimulus but one which results in a low incidence of aneuploidy. However, the studies above would seem to suggest that the observed retardation in haploid development noted in my study is not an effect of aneuploidy, but a real effect of haploidy, and I am confident that my results demonstrate that it is this that influences the rate of preimplantation development observed in this study.

I believe my results confirm those of others and demonstrate that haploidy is detrimental to normal preimplantation development, since a slower cleavage rate results. Consequently, at the time of implantation, haploid parthenogenones will have a reduced cell number compared to that found in diploid embryos and, as a result, will have less of a chance to form a viable blastocyst. My results also show that, at least during preimplantation development, embryos whose cells possess two maternally-derived haploid genomes are not at a disadvantage in terms of cell number and achievement of blastocyst stages compared to fertilized diploid embryos. It is apparent, therefore, that the presence of a paternally-derived genome is not essential for implantation and a diploid genome is superior to a haploid genome in terms of cleavage rate. However, diploid parthenogenones only reach the forelimb bud stage and die shortly thereafter. Since this is the time when a greater dependence on the extra-embryonic tissues occurs, the restricting factor for parthenogenones here is likely to be the lack of a paternal genome which is essential for the development of these tissues (Barton et al., 1984).

## CHAPTER 4

### THE CLEAVAGE RATES OF DIANDRIC AND DIGYNIC TRIPLOID MOUSE EMBRYOS DURING THE PREIMPLANTATION PERIOD

#### 4.1 INTRODUCTION

There is little informative data on the preimplantation development of triploid embryos, yet their early postimplantation development has been reasonably well documented in laboratory animals (Fischberg and Beatty, 1952; Vickers, 1969; Wróblewska, 1971; Takagi and Oshimura 1973; Takagi and Sasaki, 1976; Baranov, 1976; Kaufman and Speirs, 1987; Kaufman et al., 1989b, c; Speirs and Kaufman, 1989 a,b; and for recent reviews of triploidy in mice see Dyban and Baranov, 1987; Kaufman, 1991b) and in humans (Carr, 1971a, b; Niebuhr, 1974; Beatty, 1978; Jacobs et al., 1982).

The postimplantation development of diandric and digynic triploid mouse embryos are different (for review, see Kaufman, 1991b). However, while there are morphological differences, both types of triploids are capable of surviving up to the forelimb bud stage and can possess about 25 pairs of somites, but they are invariably smaller than diploid embryos of a similar developmental age. The variation in the phenotype achieved between diandric and digynic triploid mouse embryos, and their smaller size than developmentally matched control diploid embryos, may arise, at least in part, from an abnormally slow rate of cellular proliferation during the early postimplantation period, possibly as a consequence of their genetic imbalance. To date, however, no detailed analysis has been made which would allow a comparison to be made between the cleavage rate of these two classes of triploids to examine this. It is also unclear whether a triploid constitution invariably results in a slower cleavage rate during the preimplantation period compared to a diploid constitution, which might partly explain their poor developmental potential during the postimplantation period. This represents the basis for the present study.

## **4.2 MATERIALS AND METHODS**

### **POSTIMPLANTATION DEVELOPMENT**

8- to 12-week-old F1 hybrid female mice were superovulated with PMSG and hCG at 1500h and caged with fertile homozygous Rb(1.3)1Bnr male mice. Early pronucleate-stage fertilized eggs were isolated and diandric triploids were produced as described previously. The tripronucleate diandric triploid embryos were then transferred unilaterally to the oviducts of recipients on the first day of pseudopregnancy. The recipients were autopsied at about midday on the 9th -12th day of gestation, and the number of implantation sites present, resorptions and embryos recovered was noted. Cytogenetic analyses of the extra-embryonic membranes - principally the yolk sac- or of the intact conceptuses were made in order to confirm that they were indeed diandric triploid embryos.

In a control group, the male pronucleus of fertilized 1-cell embryos was removed via the enucleation/injection pipette and then re-inserted back into the perivitelline space where, after electrofusion, it subsequently fused to the cytoplasm to restore the diploid status of these embryos. These micromanipulated diploid embryos were then transferred to pseudopregnant recipients and isolated at day 17 of gestation when their crown-rump length and weight was measured. A further group of F1 mice were mated to Rb(1.3)1Bnr and were autopsied on day 17 of gestation when their lengths and weights were measured.

### **PREIMPLANTATION DEVELOPMENT**

A further group of diandric, and a group of digynic triploid embryos were produced and transferred separately to pseudopregnant recipients. At various times between 48-100h after hCG, the recipients were killed and the embryos recovered and transferred to colcemid for a few hours, as described previously. In all instances, note was taken of the gross morphological appearance of individual embryos at this time, whether they were at cleavage stages,

precompacted or compacted morulae or blastocysts, and from which location within the reproductive tract they were recovered. Air-dried preparations were made of these embryos and accurate cell counts made.

In each of the following control series, F1 hybrid females were mated to homozygous Rb(1.3)1Bnr males.

#### CONTROL SERIES (i)

The preimplantation development of the micromanipulated diploid embryos was analysed. After transfer to pseudopregnant recipients, they were isolated at specific time points after the HCG injection when their total cell number was established as described previously.

#### CONTROL SERIES (ii)

In this series, I wished to investigate whether incubation of early embryos in cytochalasin D and colcemid had any detrimental effect on cleavage rate, as exposure to these agents invariably occurs during nuclear manipulation. Pronucleate stage fertilized embryos were isolated from mice superovulated with PMSG and hCG at 1500h and mated, as described above. The embryos were incubated in drops of M2 supplemented with 1µg/ml of cytochalasin D and 1µg/ml of colcemid for 1.5-2h. They were then washed 4 times in M16 and after 1 hour were then transferred to recipients. At specific time points after the hCG injection, they were recovered and total cell counts made.

#### CONTROL SERIES (iii)

This series was a control for the surgical procedure of transferring embryos into pseudopregnant recipients. Pronucleate stage fertilized embryos were recovered, as described above, and retained in M16 in an incubator for 3-4 hours. They were then transferred unilaterally to the oviducts of recipients, and at specific times later they were isolated and their total cell counts established.

## 4.3 RESULTS

### 4.3.1 POSTIMPLANTATION DEVELOPMENT

#### DIANDRIC TRIPLOID MOUSE EMBRYOS

The findings in relation to the postimplantation development of diandric triploid mouse embryos in this study are presented in Table 4.1. The most advanced embryos were recovered on day 11.5 p.c., and of these, 10 were partially "turned" or "turned" early forelimb bud stage embryos (i.e. trunk region of the embryo has undergone axial rotation), each of which possessed about 25-30 pairs of somites. No morphologically normal embryos were recovered after this time, only embryonic and extra-embryonic remnants were seen. Diandric triploidy was confirmed cytogenetically in 89.5% of all of the embryos recovered. A representative metaphase spread of a diandric triploid genotype from the extra-embryonic membranes of an embryo from this series is shown in Figure 4.1.

#### MANIPULATED DIPLOID CONTROL EMBRYOS

A total of 15 manipulated diploid control embryos, transferred into 2 recipients, were left to develop into the postimplantation period. At day 17 of gestation the recipients were autopsied and 8 implants were found and 7 of these implants contained developing embryos. The embryos were morphologically normal and alive. Their mean crown-rump lengths and weights were found to be  $16.7 \pm 0.5\text{mm}$  and  $649.1 \pm 24.8\text{g}$  respectively. The same measurements made on 10 fertilized embryos developing exclusively *in vivo*, for comparison were  $16.6 \pm 0.12\text{mm}$ , and  $715 \pm 19\text{g}$  respectively. There was no significant difference between the measurements made on these two groups of diploid embryos ( $p > 0.05$ ).



**Table 4.1**    The postimplantation development of diandric triploid embryos following their transfer to recipients.

| Day of gestation<br>at autopsy(d.p.c) | No. recipients | No. embryos<br>transferred | No. implants | No. resorptions | No. embryos | Diandric triploidy<br>confirmed* | Partially "turned"/<br>"turned" embryos |
|---------------------------------------|----------------|----------------------------|--------------|-----------------|-------------|----------------------------------|---|
| 8.5                                   | 3              | 27                         | 18           | 10              | 8           | 6                                | 0                                       |
| 9.5                                   | 4              | 35                         | 32           | 19              | 13          | 12                               | 2                                       |
| 10.5                                  | 3              | 19                         | 18           | 11              | 7           | 6                                | 3                                       |
| 11.5                                  | 10             | 98                         | 62           | 42              | 20          | 19                               | 10                                      |

\* One additional embryo was a digynic triploid, another was a diploid and I was unable to analyse three further embryos.

The overall % of mice becoming pseudopregnant after surgical transfer of diandric triploid embryos during the postimplantation period was 75%.

**Figure 4.1**

A representative metaphase spread from the extra-embryonic membranes of a diandric triploid conceptus. 58 chromosomes are present, two of which are the large paternally-derived metacentric Rb(1.3) "marker" chromosomes (arrow heads). The presence of these two "marker" chromosomes provides unequivocal evidence of the diandric triploid status of this conceptus. (scale bar = 15 $\mu$ m)



#### 4.3.2 PREIMPLANTATION DEVELOPMENT

There are five groups in this study, namely a diandric triploid group, a digynic triploid group and three different controls. Cell doubling times are given for each group and a comparison is also made between groups in order to rule out the possible effects of specific technical factors on their cleavage rates. The location and morphological appearance of these embryos at the various time points studied is also described.

##### 4.3.2.1 CELL DOUBLING TIMES

After the embryos from each group had been isolated from the recipients at specific times after the original hCG injection, their total cell count was established. The values obtained were converted into natural logarithms and plotted against time after hCG. Regression lines were drawn and this allowed a cell doubling time for each group to be established.

The diandric triploid embryos had a cell doubling time of 13.55h ( $\pm 1.25$ ) between 49-100h after the original hCG injection. For the digynic triploid embryos this was 14.84h ( $\pm 1.19$ ) between 48-96h after HCG. Micromanipulated diploids (control series i) had a cell doubling time of 12.12h ( $\pm 1.16$ ) between 55-102h after hCG. Embryos incubated briefly in cytochalasin D and colcemid (control series ii) had a cell doubling time of 10.87h ( $\pm 0.75$ ) between 49-101h, and for the embryos isolated and incubated only in M16 (control series iii) this was 12.43h ( $\pm 0.74$ ) between 48-105h after hCG.

The regression lines plotted for these embryos showed that diandric and digynic triploid mouse embryos, produced by the technique of nuclear manipulation, divide at the same rate as each other ( $p > 0.05$ ) and at the same rate as the micromanipulated controls ( $p > 0.05$ ). What is also evident is that the stresses of nuclear manipulation on an embryo do not appear to detrimentally affect its cleavage rate (comparison of control series i and iii). The lines of regression plotted for these two groups were not significantly different from each other ( $p > 0.05$ ). A comparison of embryos which had briefly been exposed to cytochalasin D and colcemid with embryos that had not (control series ii and iii) were similarly not significantly different ( $p > 0.05$ ). The

findings from this study are summarised in Tables 4.2. and 4.3. Representative air-dried preparations of preimplantation diandric and digynic triploid embryos are shown in Figure 4.2, and the regression lines presented in Figure 4.3.

#### 4.3.2.2 LOCATION AND MORPHOLOGY OF PREIMPLANTATION EMBRYOS

The diandric triploids at 72h after the hCG injection were found exclusively in the oviduct and were all either precompacted or compacted morulae. By 96h, some had reached the blastocyst stage having a mean cell number of 17.92 ( $\pm$  1.16), and were located in both the oviduct and uterus. By 100h, 58% were blastocysts, located mostly in the uterus. A proportion of the blastocysts were zona-free.

At 71h after the hCG injection, the digynic triploids were found exclusively in the oviduct and were all either precompacted or compacted morulae. This was also the case at 78h. By 90h, however, some had progressed to the blastocyst stage. A small proportion of the latter were zona-free, and were located in both the oviduct and uterus. The mean cell number at 90h was 15.28 ( $\pm$  1.06). By 96h, about 45% of the embryos recovered were at the blastocyst stage and these were mostly located in the uterus. A proportion of these blastocysts were zona-free (31.4%). Because preliminary experiments had indicated that the efficiency of recovery of triploid embryos at points after 96h after the hCG injection was relatively poor, such groups were not studied.

In the various control groups studied, the micromanipulated diploid controls (series I) followed a similar progress to the triploids but by 102h after hCG only 41% were blastocysts. Nearly 50% of the embryos exposed briefly to cytochalasin D and colcemid (control series II) were blastocysts after 96h and by 101h after hCG approximately 71% had reached that stage. In the

**Table 4.2 The preimplantation development of diandric and digynic triploid mouse embryos.**

| Group                 | Time of isolation<br>(hours post hCG) | No.embryos transferred | No.embryos recovered<br>(total) healthy (%)healthy | No.analysed to establish cell number | Mean cell number<br>(±SEM) |
|-----------------------|---------------------------------------|------------------------|--|--------------------------------------|----------------------------|
| (i) Diandric triploid | 49                                    | 21                     | (21) 21 (100)                                      | 21                                   | 2.00 (±0)                  |
|                       | 72                                    | 57                     | (62)* 51 (82.2)                                    | 45                                   | 5.73 (±0.29)               |
|                       | 80                                    | 60                     | (66)* 44 (66.7)                                    | 41                                   | 9.46 (±0.65)               |
|                       | 96                                    | 78                     | (78) 56 (71.8)                                     | 48                                   | 17.92 (±1.16)              |
|                       | 100                                   | 63                     | (59) 45 (76.3)                                     | 35                                   | 30.77 (±2.21)              |
| (ii) Digynic triploid | 48                                    | 12                     | (10) 10 (100)                                      | 10                                   | 2.00 (±0)                  |
|                       | 65                                    | 59                     | (51) 45 (88.2)                                     | 41                                   | 6.00 (±0.31)               |
|                       | 71                                    | 55                     | (55) 47 (85.4)                                     | 37                                   | 6.05 (±0.31)               |
|                       | 78                                    | 51                     | (43) 37 (86.0)                                     | 36                                   | 9.47 (±0.58)               |
|                       | 90                                    | 86                     | (63) 46 (73.0)                                     | 39                                   | 15.28 (±1.06)              |
|                       | 96                                    | 77                     | (60) 50 (83.3)                                     | 40                                   | 23.20 (±1.80)              |

\* It is assumed that these embryos must have resulted from the parthenogenetic activation of the recipients' eggs, due to the activating stimulus of the anaesthetic employed during the transfer procedure (Kaufman, 1975; Kaufman and Sachs, 1975).

The overall % of mice becoming pseudopregnant after surgical transfer of diandric and digynic triploid embryos was 91.2% and 91.1% respectively.



**Table 4.3 The preimplantation development of control diploid mouse embryos.**

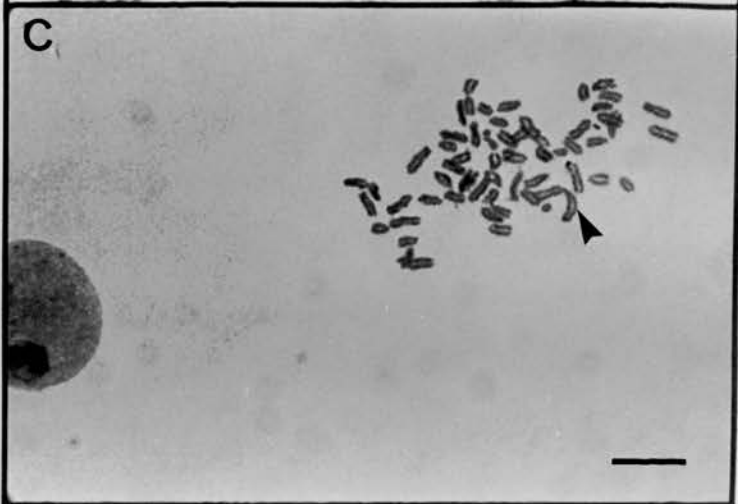
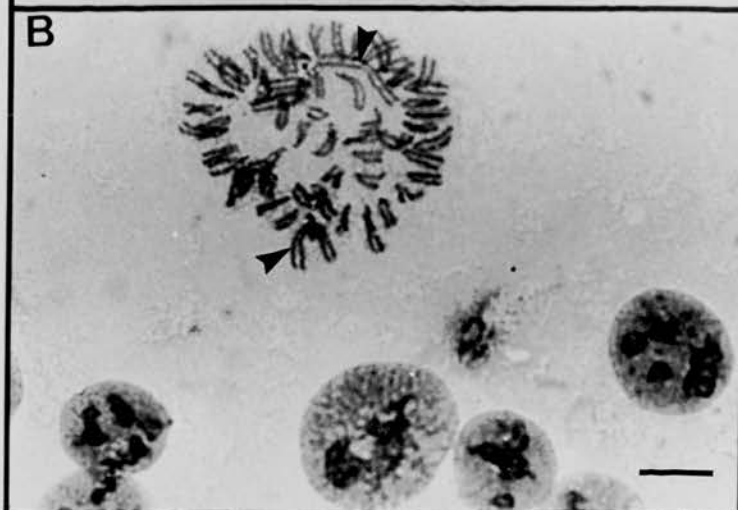
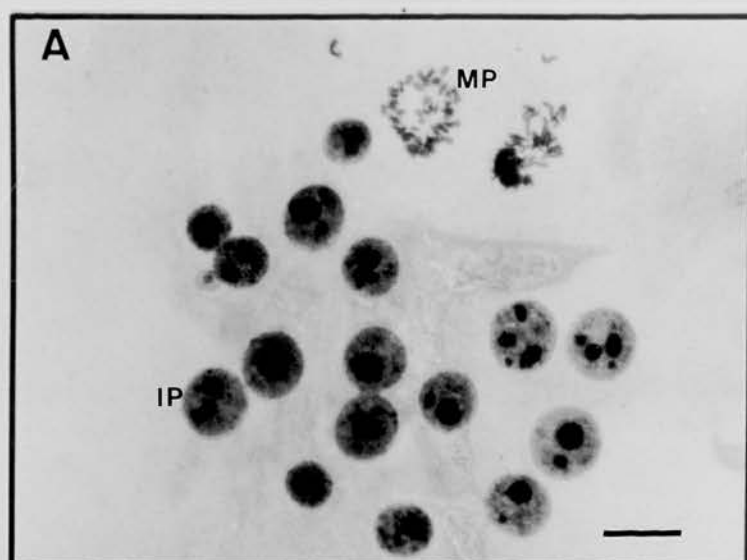
| Group  | Time of isolation<br>(hours post hCG <sub>i</sub> ) | No.embryos<br>transferred | No.embryos<br>recovered<br>(total) healthy (%)healthy | No.analysed<br>to establish<br>cell number | Mean cell<br>number<br>(±SEM) |
|--|---|---------------------------|---|--|-------------------------------|
| (i) Micromanipulated<br>diploid control  | 55  | 20                        | (26)* 21* (80.8)                                      | 19   | 2.26 (± 0.15)                 |
|  | 72  | 34                        | (40)* 37* (92.5)                                      | 31   | 5.55 (± 0.33)                 |
|  | 79  | 44                        | (50)* 41 (82.0)                                       | 30   | 9.60 (± 0.77)                 |
|  | 96  | 60                        | (53) 39 (73.6)  | 34   | 22.62 (± 1.65)                |
|  | 102   | 53                        | (50) 39 (78.0)  | 39   | 33.30 (± 3.04)                |
| (ii) Cytochalasin D/<br>colcemid<br>controls   | 49  | 31                        | (34)* 25 (73.5)                                       | 25   | 2.00 (± 0)                    |
|  | 74  | 51                        | (49) 46 (93.9)  | 40   | 7.40 (± 0.44)                 |
|  | 80  | 47                        | (55)* 49* (89.1)                                      | 44   | 12.79 (± 0.47)                |
|  | 96  | 41                        | (51)* 46* (90.2)                                      | 41   | 31.34 (± 1.55)                |
|  | 101   | 64                        | (59) 51 (86.4)  | 48   | 55.46 (± 2.84)                |
| (iii) Transferred<br>1-cell controls<br>(not exposed to<br>cytoskeletal<br>inhibitors) | 48  | 58                        | (68)* 61* (89.7)                                      | 58   | 2.22 (± 0.08)                 |
|  | 75  | 55                        | (62)* 51 (82.2)                                       | 46   | 6.46 (± 0.51)                 |
|  | 80  | 70                        | (83)* 56 (67.5)                                       | 50   | 13.74 (± 0.67)                |
|  | 96  | 65                        | (65) 53 (81.5)  | 41   | 30.51 (± 2.44)                |
|  | 105   | 106                       | (83) 57 (68.7)  | 41   | 51.90 (± 3.32)                |

\* It is assumed that the additional embryos recovered over and above those transferred must have resulted from the parthenogenetic activation of the recipients' eggs, due to the activating stimulus of the anaesthetic employed during the transfer procedure (Kaufman, 1975; Kaufman and Sachs, 1975).

The overall % of mice becoming pseudopregnant after surgical transfer of micromanipulated diploid control embryos, cytochalasin D/colcemid embryos and 1-cell controls was 95.2%, 100% and 77.8% respectively.

**Figure 4.2**

- A) Representative air-dried preparation of a preimplantation triploid embryo. This embryo has 18 cells (16 interphase nuclei (IP) and two metaphase plates (MP)) (low magnification). (scale bar = 40 $\mu$ m)
- B) High power view of a diandric triploid metaphase plate of a preimplantation embryo. The presence of 58 chromosomes of which two are "marker" chromosomes (arrow heads) provides unequivocal evidence of the diandric triploid status of this embryo. (scale bar = 12.5 $\mu$ m)
- C) High power view of a digynic triploid metaphase plate of a preimplantation embryo. The presence of 59 chromosomes of which only one is a "marker" chromosome (arrow head) provides unequivocal evidence of the digynic triploid status of this embryo. (scale bar = 14 $\mu$ m)

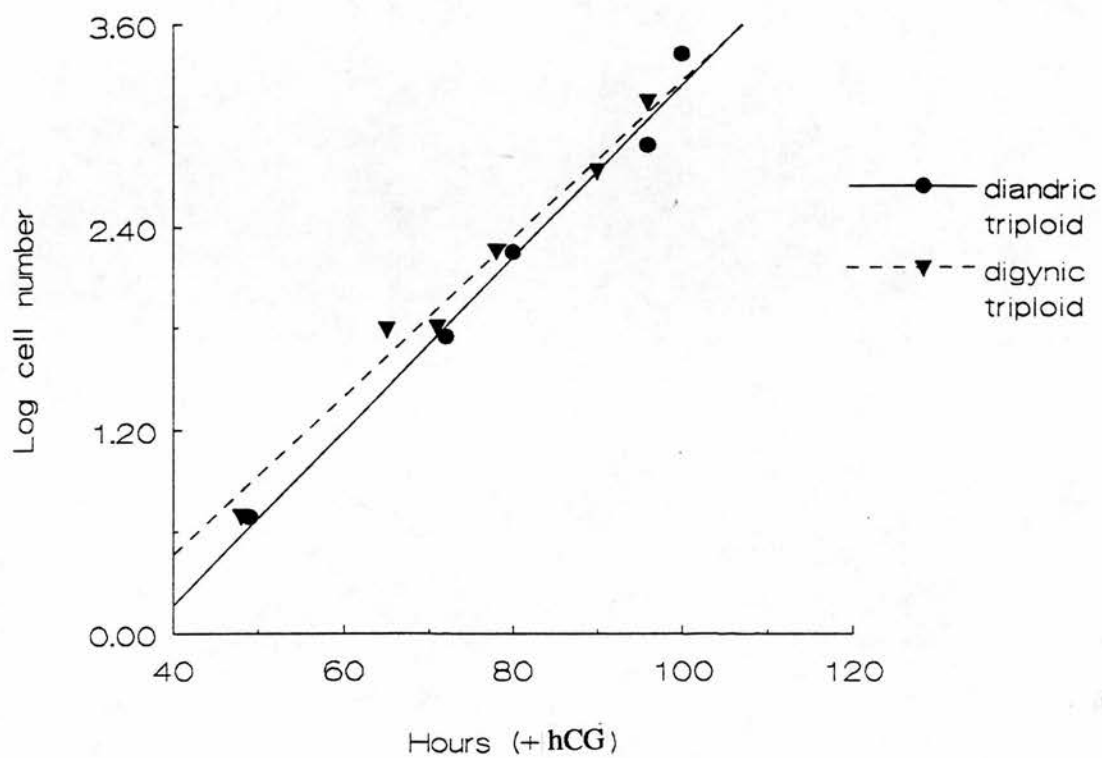


### Figure 4.3

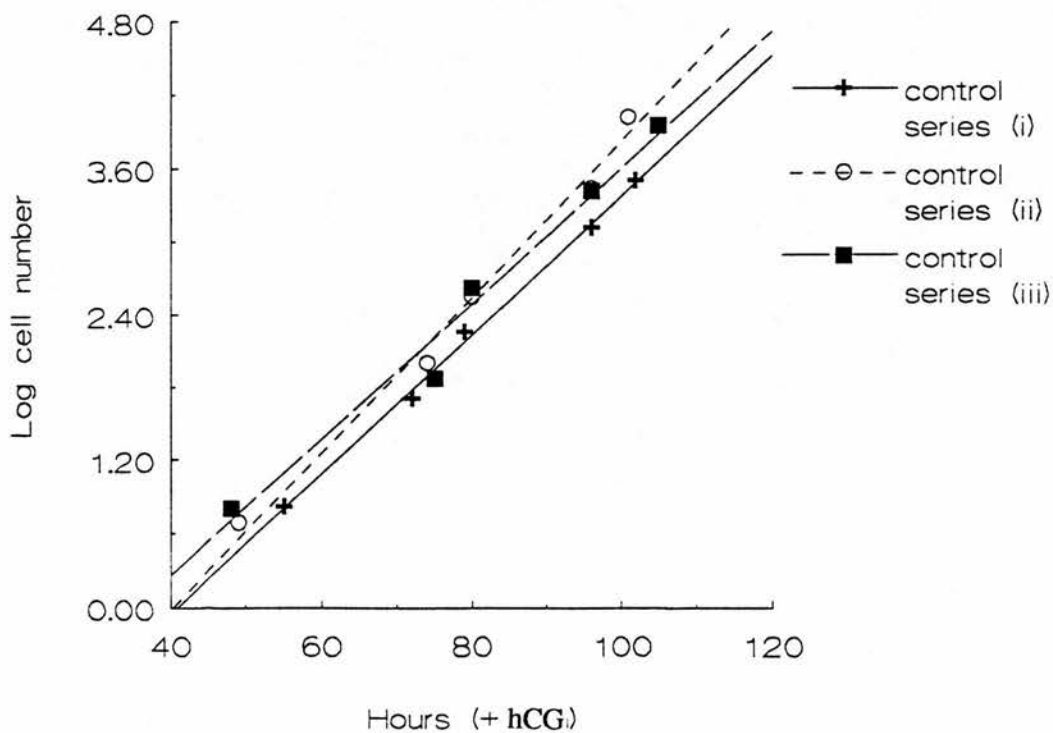
Log total cell number of embryos isolated at various times during the preimplantation period, plotted against time after hCG injection to induce ovulation in (a) diandric and digynic triploid mouse embryos, (b) micromanipulated diploid control embryos (control series (i)), diploid control embryos exposed to cytochalasin D and colcemid (control series (ii)), and diploid control embryos not exposed to cytochalasin D and colcemid (control series (iii)). By regressing the log cell number against time, it was possible to estimate the gradient of each line using least squares. The regression was weighted using the sample size for each mean value. From the gradient, it was possible to estimate the average cell doubling time for each series. No significant difference was found between the cell doubling times of diandric and digynic triploid embryos and the micromanipulated controls (control series (i)). Similarly, no significant difference was found to exist between the cell doubling times of control series (i) and control series (ii), nor between control series (ii) and control series (iii) (see text for further details).

Figure 4.3

(a)



(b)



additional control group (control series III) 23% were blastocysts by 96h and were located in both the oviduct and the uterus. By 105h, all the embryos were located in the uterus and nearly 66% of these were blastocysts. The findings from this component of the study are presented in diagrammatic form in Figure 4.4.



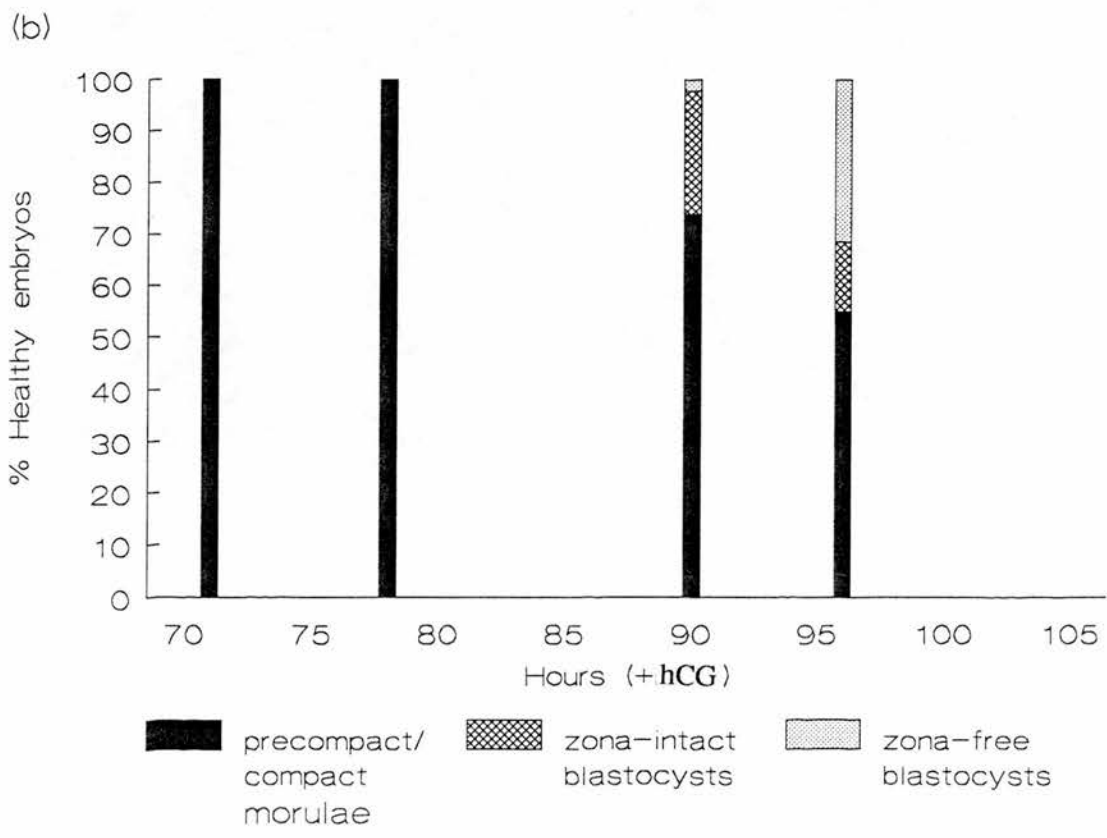
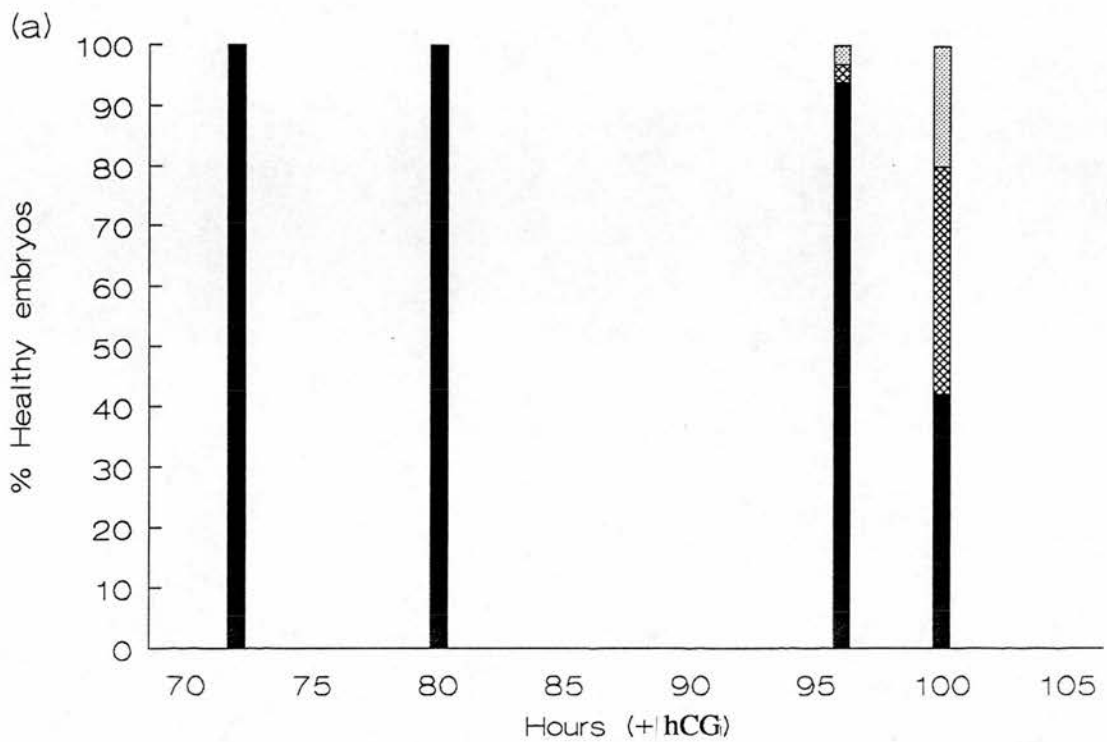
**Figure 4.4**

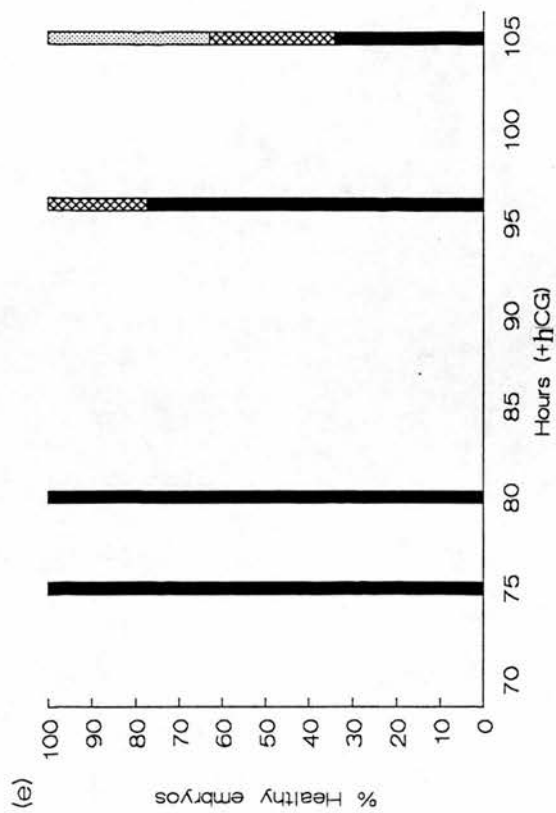
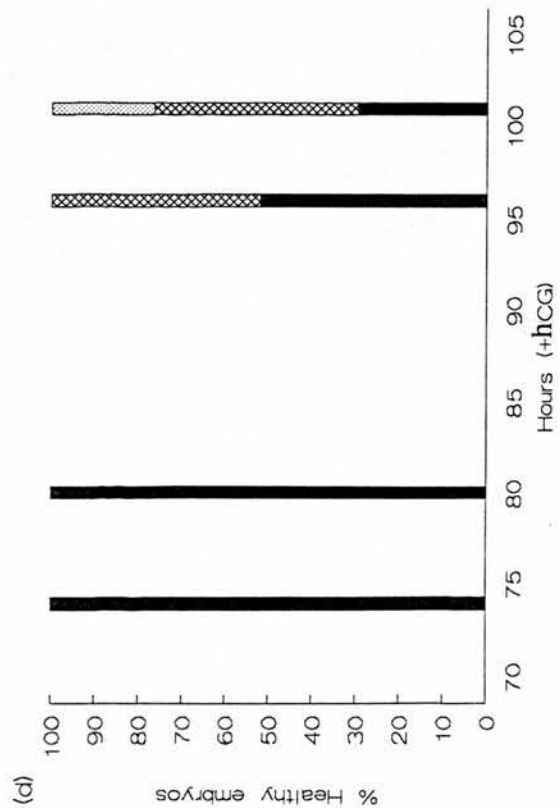
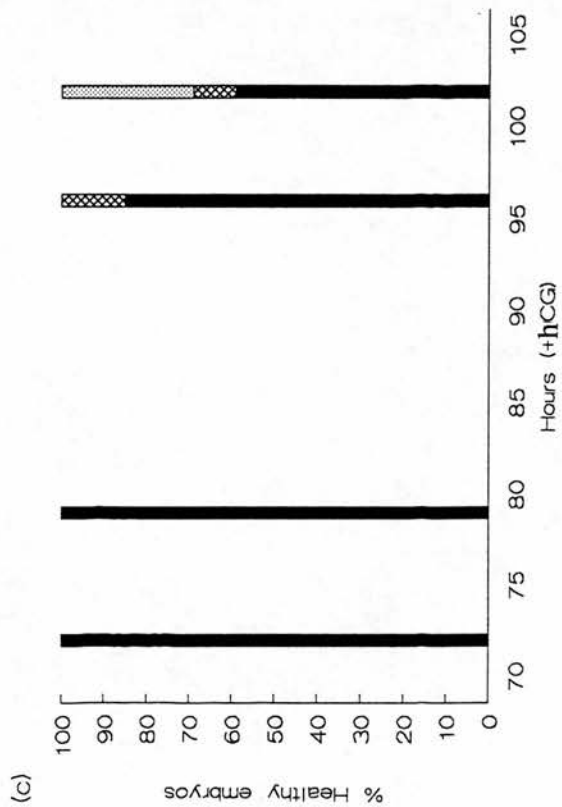
Morphological appearance of embryos isolated at various times during the preimplantation period plotted against time after hCG to induce ovulation.

- a) diandric triploid embryos.
- b) digynic triploid embryos.
- c) micromanipulated diploid control embryos (control series (i)).
- d) diploid control embryos exposed to cytochalasin D and colcemid (control series (ii)).
- e) diploid control embryos not exposed to cytochalasin D and colcemid (control series (iii)).

(morphological appearance based on number of recovered healthy embryos from Table 4.2 and Table 4.3)

Figure 4.4





#### 4.4 DISCUSSION

##### *The preimplantation development of diandric and digynic triploid mouse embryos*

My results clearly demonstrate that diandric and digynic triploid mouse embryos cleave at the same rate as control diploid embryos during the preimplantation period. In addition, I have also shown that the cleavage rates of diandric and digynic triploid embryos during this time are the same. An additional haploid set of male or female chromosomes, in addition to those ordinarily present in a fertilized diploid embryo, has no obvious detrimental effect on cleavage rate during the preimplantation period, while the absence of a haploid set of chromosomes clearly does (Modliński, 1975, 1980; Kaufman and Sachs, 1976; Kaufman, 1981; Henery and Kaufman, 1992a). This will, I believe, settle the confusion surrounding the effect that a triploid genome has on early development. While some authorities have reported that triploid mouse embryos develop at the same rate as controls (Niemierko, 1975; Funaki, 1981), others have reported that they have a slower rate of cleavage (Beatty and Fischberg, 1951; Takagi and Sasaki, 1976).

The overall implantation rate of diandric triploid mouse embryos in this experiment was about 73%. This rate demonstrates that the majority of diandric triploid embryos successfully reached the blastocyst stage and were able to induce a decidual reaction. This implantation rate compares favourably with that observed by Kaufman et al. (1989b, c) on day 10 of gestation (9.5 days p.c.). From an analysis of the morphological appearance of diandric triploid embryos in my study, it appears likely that they were healthy at least up until day 11.5 p.c.. However, the diandric triploid embryos appeared to be smaller in size than controls at a similar developmental stage, and this observation is consistent with previous findings (Kaufman et al., 1989b, c). This is similar to that which is found with postimplantation digynic triploid mouse embryos (Surani and Barton, 1983; Kaufman and Speirs, 1987; Kaufman et al., 1989b). Earlier observations on blastocyst diameter in rabbits noted that triploids were consistently smaller than diploids (Bomsel-Helmreich, 1965), though whether this finding bears any relationship to their postimplantation development is unclear. Since blastocyst diameter was not measured in

the present study, I am unable to comment on the significance of this observation. However, I believe that an analysis of cell number may be a better indication of developmental stage in preimplantation triploid embryos, though a more relevant source of information might be the ratio of inner cell mass (ICM) cells to total cells in these blastocysts.

My study, unlike those of, for example, Beatty and Fischberg (1951), Edwards (1958b) and Baranov (1976), is based on large numbers of embryos, and consequently allows critical statistical analyses to be performed. I have also been able to rule out any possible effects of technical (i.e. experimentally-induced) stresses on the diandric and digynic triploid embryos by using a comparison with appropriate controls. I have found that handling of the embryos, even for just a short period of incubation in tissue culture medium before transferring them to recipients, has an adverse effect on cleavage rate, and a control was therefore provided which took this aspect into account. Suitable controls were also undertaken to exclude the micromanipulatory and electrofusion procedures. Nuclear manipulation is not detrimental to embryonic survival (Evsikov et al., 1990) and, in fact, one recent study on manipulated mouse preimplantation embryos suggested that the drilling of a large opening (rather than a small one) in the zona facilitates hatching of the blastocyst and significantly improves their developmental potential (Cohen and Feldberg, 1991). I was also able to show, in an additional control group, that a brief incubation in cytoskeletal inhibitors has no obvious detrimental effect on cell number during the preimplantation period (see also Siracusa et al., 1980; Evsikov et al., 1990).

I have been able to examine the preimplantation development of both diandric and digynic triploid embryos using the same experimental technique to induce triploidy. Thus, I am able to rule out possible technical influences on their cleavage rate. While micromanipulatory techniques to produce digynic triploid embryos may have been technically more difficult than, for example, inducing second polar body retention, I believed it was necessary in order to

allow a valid comparison of these two genotypes. Although this may have resulted in a greater degree of heterozygosity than would have occurred following suppression of the second polar body, I believe this factor has probably a negligible effect on cleavage rate.

I have shown that micromanipulated diploid fertilized embryos can develop normally and reach the blastocyst stage. It is possible, however, that they too fail, like triploids, to reach term. To rule out the possibility that *in vitro* culture and micromanipulation may have a long term detrimental effect, and may partly explain why experimentally-induced triploid embryos fail to develop to term, a few micromanipulated diploid embryos were allowed to develop late into the postimplantation period. Although only about half of the embryos implanted, a high proportion of the latter were recovered as healthy morphologically normal-looking diploid embryos at day 17 of gestation. By this means, I was able to demonstrate that these procedures do not themselves cause abnormal development and premature death. Reports have suggested that superovulation, *in vitro* culture and micromanipulation may each have a long term detrimental effect on embryonic development, resulting in prolonged gestation, small litters and increased birth weights (Biggers and Papaioannou, 1991; Willadsen et al., 1991; Walker et al., 1992). While I did not measure birth weights, I found that the mean crown-rump lengths and weights of the micromanipulated diploid control embryos that were analysed on day 17 of gestation were within the normal range, and not significantly different, from similar measurements made on non-micromanipulated diploid embryos from naturally ovulating F1 female mice. It is possible, however, that the detrimental influence of these technical procedures may only become apparent after birth, being too small to be detected during the postimplantation stages of development.

The fusigenic stimulus used in this study was electrofusion. This stimulus has been shown to be very effective for pronuclear transplantation of mouse eggs, with high rates of development of reconstituted embryos to the blastocyst stage (Tsunoda et al., 1987; Kono and Tsunoda, 1988). I believe that this is technically superior to the use of inactivated Sendai virus for the fusion of karyoplast to cytoplasm if the problems associated with the production and



maintenance of Sendai virus are taken into account. Also, improved rates of postimplantation development of mouse tetraploids have recently been achieved when electrofusion has been used as a fusigenic stimulus (Kaufman and Webb, 1990) rather than inactivated Sendai virus (O'Neill et al., 1990). An improved degree of postimplantation development of triploid embryos might, therefore, also be expected to result from the use of this fusigenic stimulus.

Thus, I am confident that my results show that the cleavage rate of diandric and digynic triploid embryos do not significantly differ from each other or from that of the various controls used in this study, and that their rate of achievement to the morula and blastocyst stage also appears to be similar. By contrast, most haploid embryos appear to be unable to cavitate normally (Modliński 1975, 1980; Kaufman, 1981, 1983; Henery and Kaufman, 1992a). It is still not clear what influence an altered nuclear-cytoplasmic ratio might have on development because a decrease in this ratio is detrimental to preimplantation development, as is evidently the situation in the case of haploids (Modliński, 1975, 1980; Opas, 1977; McGrath and Solter, 1986; Surani et al., 1986; Henery and Kaufman, 1992a). An increase in the nuclear-cytoplasmic ratio, however, appears not to be detrimental for triploids to achieve the morula and blastocyst stages at about the same time as controls. It is of interest that Evsikov et al. (1990) increased the nuclear-cytoplasmic ratio of diploid embryos by the removal of about one third of the cytoplasm, and this manoeuvre appeared to have the effect of reducing the cleavage rate of this group. However, it is relevant to note that technical difficulties have been encountered in association with the removal of the female but not the male pronucleus, and that this is likely to partly explain why gynogenetic haploids produced by micromanipulation develop better than androgenetic haploids, despite the fact that they have an identical nuclear-cytoplasmic ratio (Modliński, 1975).

#### *The extra-embryonic tissues in diandric and digynic triploid mouse and human conceptuses*

Diandric triploidy is worthy of investigation because it represents the genetic basis of the partial form of hydatidiform mole in human conceptions. Unfortunately, this chromosome constitution in the mouse does not truly reflect the situation in humans because diandric

triploid mouse embryos do not exhibit obvious molar changes in their extra-embryonic tissues. Although this is the case, the histology of some intact decidua which contained diandric triploid mouse embryos appeared to show an abnormally small embryo associated with an excessively large amount of extra-embryonic membranes (see also Barton et al., 1984). It is possible, therefore, that the influence of the additional paternally-imprinted genes in diandric triploid mouse embryos may be towards directing this excessive growth of their extra-embryonic membranes, since this feature is not characteristically seen in relation to the extra-embryonic tissues of digynic triploid mouse embryos. A greater proportion of cells than is normally found in association with diploid embryos may be found in the trophoctoderm of the triploid blastocysts, and proportionately less may be present in their ICM (which develops into the embryo proper (Gardner, 1972)), and this may partly explain the smaller size of these embryos during the postimplantation period.

Complete hydatidiform moles in humans have characteristically an exclusively paternally-derived genome. Unfortunately, there is only a relatively small amount of information on the preimplantation development of these conceptuses, most of which is derived from *in vitro* fertilization studies (Edwards et al., 1992). Very few diploid mouse androgenones reach the blastocyst stage (Barton et al., 1984; Kaufman et al., 1989a), and their development to this stage, when it occurs, is retarded (Surani et al., 1986; Latham and Solter, 1991; Mann and Stewart, 1991; Kono et al., 1993). It is clear, therefore, that the development of embryos which lack a maternally-derived genome is poor. It is possible that the reason for this is that it is a consequence of their having exclusively paternally-derived X chromosomes, since Thornhill and Burgoyne (1992) have recently reported a developmental lag at the egg-cylinder stage in XO mouse embryos which had a paternally-derived X chromosome. Furthermore, XO embryos, which had a maternally-derived X chromosome, were apparently significantly larger than both the  $X_p$ O embryos and the XX normal euploid fertilized embryos. Molecular studies have also suggested that the poor preimplantation development of diploid androgenones may be the result of a limited amount of certain "factors" within their cells which are only weakly produced by paternally-inherited genes (Hagemann and First, 1992). These authors found that

injecting cytoplasmic extracts of either parthenogenetic or fertilized embryos into 4-cell androgenones led to an increase in the number that achieved the blastocyst stage. If this finding is confirmed, one possible explanation why diandric triploids are able to develop successfully during this period may be because of the presence of maternally-derived genes within their genotype which are able to produce these "factors" in sufficient concentrations that allow the majority of embryos to reach the blastocyst stage.

*Postimplantation phenotypes of diandric and digynic triploid mouse embryos*

Despite the fact that during the preimplantation period digynic and diandric triploid mouse embryos are morphologically remarkably similar, the postimplantation morphological appearance of these two classes of embryos are quite different. The digynic triploid embryos characteristically display neural tube and often cardiac abnormalities, while the diandric triploids, though smaller than developmentally matched controls, are nevertheless usually morphologically normal (Kaufman and Speirs, 1987; Kaufman et al, 1989b, c; Speirs and Kaufman, 1989a; Kaufman, 1991b). However, one recent report has described a triploid mouse embryo recovered at 14 days of gestation which had several distinct morphological abnormalities, including microphthalmia and small and misplaced otic primordia (Bos-Mikich and Whittingham, 1992). Although the embryo was triploid on karyotypic examination, it was unclear whether triploidy was of diandric or digynic origin, although the former was presumed to be the most likely. If this is the case, the morphological differences observed between this diandric triploid embryo and the ones examined by Kaufman et al. (1989b, c) and in this study, may simply reflect strain differences.

Much information has accumulated over the last few years regarding the different, but complementary, roles of the maternal and paternal genomes during mammalian embryonic development (Mann and Lovell-Badge, 1984; McGrath and Solter, 1984; Barton et al., 1984; Surani et al., 1984). These complementary roles are believed to be a result from epigenetic modifications of some parental alleles which give rise to functional differences between certain homologous chromosomal regions (Cattanach, 1986; Solter, 1988; Surani, 1991). A

duplication of one set of parental genes with a corresponding deficiency in the other may result in different phenotypic traits within the embryo brought about through changes in the balance of gene dosage required for normal development (Epstein, 1986; Barton et al., 1991).

The different phenotypes exhibited by an excess of one parental genome can also be seen, but to a lesser extent, in chimerics between androgenetic/fertilized embryos and parthenogenetic/fertilized embryos (Surani et al., 1988; Nagy et al., 1987, 1989; Fundele et al., 1989, 1990; Barton et al., 1991). Androgenetic/fertilized chimeric fetuses or liveborns, for example, have been described as being larger, but thinner and longer than normal fertilized fetuses/liveborns (Barton et al., 1991), and may show extensive abnormalities of the skeletal system affecting the chest and limbs (see also, Mann et al., 1990). In addition, androgenetic cells in these chimeras specifically contribute certain tissues including heart and skeletal muscle but not to the brain. In contrast to this, parthenogenetic/fertilized chimerics are retarded in growth and parthenogenetic cells in these chimeras make a substantial contribution to the brain, epidermis and germ cells but rarely to skeletal muscle. These phenotypic effects and specific distribution of cells to certain lineages suggests that the effects of imprinted genes may be lineage specific. In the case of the diandric and digynic triploid mouse embryos, any effect of duplication of imprinted genes may be diluted by the presence of the opposite genotype within the same cell.

It is not clear how early in diandric and digynic triploid development genomic imprinting effects manifest themselves, but my results strongly suggest that they do not affect the number of cells in diandric and digynic triploid embryos or the morphological appearance of these embryos during the preimplantation period. While subtle differences may exist at the molecular level, it is only later in triploid development that genomic imprinting effects become visibly obvious. It might be instructive, however, to look more closely at the preimplantation development of specific classes of triploids, for example, those with an XYY diandric triploid sex-chromosome constitution, since it is known that this class of triploid (by contrast to the XXX and XXY classes) develops least well after implantation (Kaufman et al., 1989c).

However, both diandric and digynic genotypes are lethal conditions in mice, and, in fact, no triploid laboratory mammal has ever reached term. In contrast, both amphibian and fish triploids can attain normal development and may even be fertile and capable of reproducing (Fankhauser, 1941; Small and Benfrey, 1987). In man, pure triploids do very occasionally reach term, but their postnatal survival is very poor. Furthermore, the incidence of this condition is unknown, since descriptions of only a very small number of conceptuses with this genotype are ever reported in the literature.

## **CHAPTER 5**

### **THE CLEAVAGE RATE OF TETRAPLOID MOUSE EMBRYOS DURING THE PREIMPLANTATION PERIOD**



## 5.1 INTRODUCTION

Despite the fact that spontaneous tetraploidy is a rare phenomenon in mice (Beatty, 1957; Dyban and Baranov, 1987), such embryos may be produced experimentally by a variety of means (see, for example Snow, 1973, 1975; Kubiak and Tarkowski, 1985; O'Neill et al., 1990; Kaufman and Webb, 1990). When tetraploid embryos are produced by inhibiting the first (or second) cleavage mitosis, or by fusing the two blastomeres of 2-cell diploid embryos together (for example, by electrofusion), the increase in ploidy is due to their having twice the normal chromosome complement. The embryos therefore possess two identical maternal and two identical paternal genetic contributions and are consequently genetically homozygous tetraploids.

Although a wide variety of techniques have been used to induce tetraploid development, the successful birth of liveborn tetraploids has only very rarely been reported and unfortunately, these liveborns were eaten soon after birth and little information was available on them (Snow, 1976). Tetraploidy in humans, in rare instances, is also compatible with live birth and such individuals can live for up to 2 years but with a range of congenital abnormalities and mental retardation (Lafer and Neu, 1988).

Recently, electrofusion has been shown to be an extremely efficient fusogenic stimulus, and has allowed high rates of early postimplantation development of homozygous tetraploidy to be achieved (Kaufman and Webb, 1990; Kaufman, 1991a, 1992a). This experimental approach has previously been applied to rabbit embryos (Ozil and Modliński, 1986), bovine embryos (Iwasaki et al., 1989), as well as to mouse embryos (Kubiak and Tarkowski, 1985; Kato and Tsunoda, 1987; Winkel and Nuccitelli, 1989), though in these studies, only preimplantation development has been achieved. Electrofusion is potentially more advantageous than some other methods used to induce tetraploidy since it is of short duration, it elicits a quick reaction, it is highly reproducible and, unlike cytochalasin B-induced tetraploidy, it does not require the

cells to be fused to be at a particular stage in their cell cycle. Furthermore, there is no evidence with this stimulus that tetraploid embryos revert to diploid/tetraploid mosaicism during development (James et al., 1992).

In the light of the successful postimplantation development so far achieved (Kaufman and Webb, 1990; Kaufman, 1991a, 1992a), I decided it was an opportune time to re-evaluate the preimplantation development of tetraploid embryos in as near to optimal conditions as possible. In particular, I was interested to establish any effect of tetraploidy on cleavage rate during the preimplantation period since it has been suggested that the developmental failure in tetraploids may be the result of a reduced proliferative ability compared to diploid cells (Tarkowski et al., 1977; Dyban and Baranov, 1987). I also wished to demonstrate that experimental procedures, even the isolation of embryos, followed by their brief incubation *in vitro* followed by their rapid return to an appropriate recipient can have a significant effect on the findings.

## 5.2 MATERIALS AND METHODS

### CONTROL SERIES (i)

Superovulated F1 hybrid female mice were mated with homozygous Rb(1.3)1Bnr males and at specific points after the HCG injection, the female mice were killed in order to recover the embryos. Air-dried preparations were made of these embryos and the preparations were stained and cell counts established.

### CONTROL SERIES (ii)

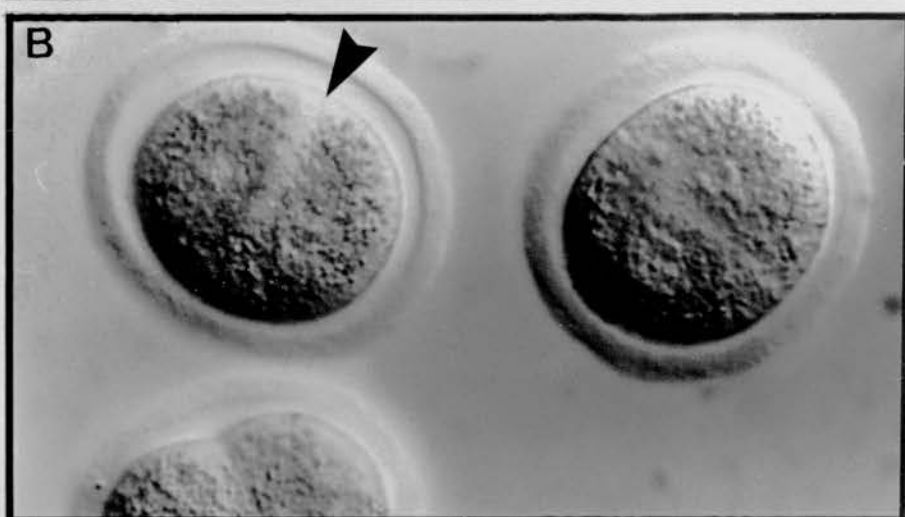
F1 hybrid female mice were superovulated and mated with homozygous Rb(1.3)1Bnr males. Early on the morning of the second day of gestation, the female mice were killed to recover the 2-cell stage embryos. These embryos were cultured for 2-3 hours, and then transferred to the oviducts of recipients on the first day of pseudopregnancy. At specific time points after the HCG injection, the recipient mice were killed in order to recover the transferred embryos from the oviduct or uterus. Air-dried preparations were made of these embryos and cell counts established.

### TETRAPLOID SERIES (iii)

F1 hybrid female mice were superovulated and mated with homozygous Rb(1.3)1Bnr males. Early on the morning of the second day of gestation, the female mice were killed to recover the 2-cell stage embryos, and these embryos were subjected to electrofusion to fuse their two blastomeres together, as described previously (see Figure 5.1a and b). After the 2 blastomeres had fused together to form 1-cell tetraploid embryos, these embryos were transferred to recipients on the first day of pseudopregnancy. At specific time points after the HCG injection, the recipients were killed and the operated oviducts or uteri flushed to recover the transferred embryos. These embryos were then transferred to colcemid for a few hours to arrest their cells in mitotic metaphase and then air-dried preparations were made to establish their ploidy and cell number, as described previously. In all instances the electrofused embryos were found to have a tetraploid constitution.

Figure 5.1

- A) Two-cell stage diploid embryos before electrofusion to fuse their two blastomeres together (DN = diploid nucleus).
- B) In each of these embryos, the two blastomeres are in the process of fusing together (arrow head), or are already fused. During the resumption of their "first cleavage division" (i.e. really the second cleavage division), the two diploid nuclei of each blastomere will undergo DNA synthesis and after nuclear membrane breakdown, the chromosomes will assemble on the spindle to form a tetraploid mitotic plate. The "first cleavage division" will result in a two-celled embryo with each blastomere containing a tetraploid nucleus (TN).
- C) Tetraploid embryos compact at the 4-cell stage instead of at the 8-cell stage which is characteristic of diploid mouse embryos.  
(scale bar = 20 $\mu$ m)



Note was also made of the gross morphological appearance of individual embryos recovered from control series (ii) and the tetraploid series (iii). These embryos were categorised as being morulae which were either precompacted or compacted, or blastocysts which were zona-intact or zona-free. Note was also taken as to whether the embryos were recovered from the oviduct or uterus.

### 5.3 RESULTS

The findings from this study may most conveniently be considered first in relation to the total cell count of diploid (control) embryos, and electrofusion-induced tetraploid embryos at specific times during the preimplantation period, and secondly in relation to the gross morphological appearance of these embryos at these times.

#### 5.3.1 CELL DOUBLING TIMES

##### (i) *IN VIVO* CELL DOUBLING TIME

When the mean value for the total cell count of embryos from control series (i) was established at various intervals after the hCG injection for inducing ovulation (see Table 5.1), these values were converted into natural logarithms and the latter were then plotted graphically against time after hCG injection (see Figure 5.2). The regression line obtained allowed a cell doubling time (in hours) to be established. The cell doubling time ( $\pm$  SEM) between 49-102 hours after the hCG injection was found to be  $10.27 \pm 0.24$  hours.

##### (ii) CELL DOUBLING TIME AFTER ISOLATION AND BRIEF CULTURE OF DIPLOID (CONTROL) EMBRYOS AT THE 2-CELL STAGE AND THEIR SUBSEQUENT TRANSFER TO PSEUDOPREGNANT RECIPIENTS.

When embryos from this group were isolated from the recipients at specific times after the hCG injection, their mean total cell count was established as in control series (i) (see Table 5.1). These values were converted to natural logarithms and the latter was then plotted graphically against time after hCG injection. The regression line obtained allowed a cell doubling time (in hours) to be established in this series, between 70-122 hours after the hCG injection (see Figure 5.2). The gradient of the calculated regression line was found to be significantly different from the value obtained from the *in vivo* study ( $p < 0.05$ ) with, in this case, a cell doubling time ( $\pm$  SEM) over the period studied of  $15.86 \pm 1.45$  hours.



**Table 5.1** Mean cell number of embryos recovered at various time after HCG injection to induce ovulation in control (*in vivo*) series (i), control (transfer) series (ii), tetraploid series (iii).

|       | Group                                       | Time of isolation<br>(hours post hCG) | No.embryos<br>transferred | No.embryos<br>recovered<br>(total) healthy (%) | No. analysed<br>to establish<br>cell number | Mean cell number<br>( $\pm$ SEM) |
|-------|---|---------------------------------------|---------------------------|--|---|----------------------------------|
| (i)   | Control<br>( <i>in vivo</i> )<br>series (i) | 49                                    | NA                        | 80   | 80  | 2.25 ( $\pm$ 0.07)               |
|       |   | 68                                    | NA                        | 56   | 56  | 8.50 ( $\pm$ 0.27)               |
|       |   | 72                                    | NA                        | 57   | 57  | 11.72 ( $\pm$ 0.58)              |
|       |   | 79                                    | NA                        | 78   | 78  | 19.21 ( $\pm$ 0.72)              |
|       |   | 96                                    | NA                        | 44   | 44  | 54.14 ( $\pm$ 2.49)              |
|       |   | 102                                   | NA                        | 39   | 39  | 78.26 ( $\pm$ 2.52)              |
| (ii)  | Control<br>(transfer)<br>series (ii)        | 70                                    | 64                        | (59) 56 (94.9)                                 | 43  | 9.28 ( $\pm$ 0.49)               |
|       |   | 82                                    | 106                       | (63) 61 (96.8)                                 | 55  | 18.09 ( $\pm$ 0.70)              |
|       |   | 95                                    | 66                        | (54) 52 (96.3)                                 | 43  | 31.58 ( $\pm$ 1.25)              |
|       |   | 100                                   | 60                        | (65)* 51 (78.5)                                | 45  | 50.40 ( $\pm$ 2.18)              |
|       |   | 110                                   | 72                        | (39) 36 (92.3)                                 | 22  | 68.50 ( $\pm$ 3.30)              |
|       |   | 122                                   | 155                       | (110) 88 (80.0)                                | 54  | 87.91 ( $\pm$ 2.39)              |
| (iii) | Tetraploids<br>series (iii)                 | 74                                    | 53                        | (51) 51 (100)                                  | 48  | 4.69 ( $\pm$ 0.24)               |
|       |   | 81                                    | 61                        | (70)* 58 (82.8)                                | 52  | 7.31 ( $\pm$ 0.20)               |
|       |   | 97                                    | 63                        | (65)* 51 (78.5)                                | 38  | 15.21 ( $\pm$ 0.46)              |
|       |   | 105                                   | 93                        | (83) 72 (86.7)                                 | 56  | 21.68 ( $\pm$ 0.83)              |
|       |   | 116                                   | 111                       | (68) 51 (75.0)                                 | 37  | 33.54 ( $\pm$ 1.80)              |

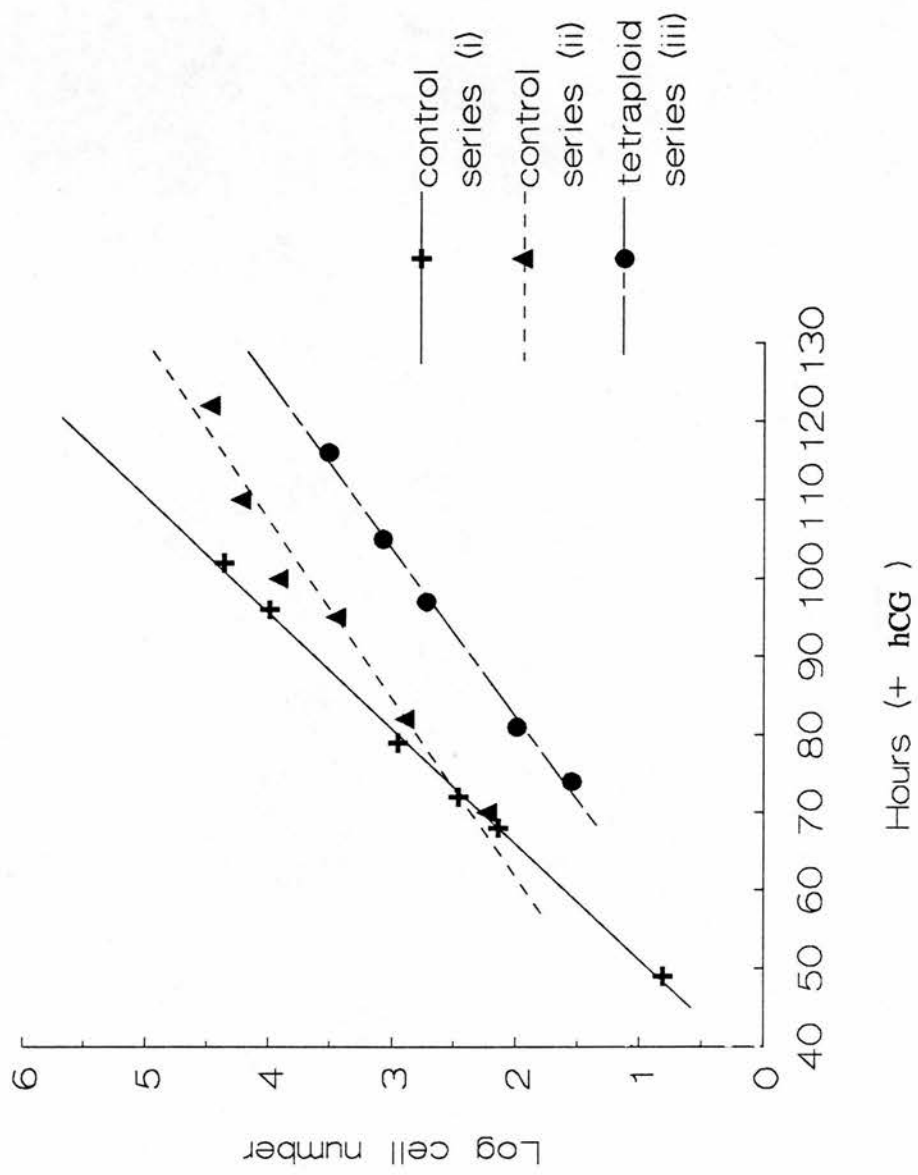
\* It is assumed that these embryos must have resulted from the parthenogenetic activation of the recipients' eggs, due to the activating stimulus of the anaesthetic employed during the transfer procedure (Kaufman, 1975; Kaufman and Sachs, 1975).

The overall % of mice becoming pseudopregnant after surgical transfer of control 2-cells and tetraploid embryos was 95% and 94.3% respectively.

### Figure 5.2

Log total cell number of embryos isolated at various times during the preimplantation period, plotted against time after hCG injection to induce ovulation in (i) *in vivo* controls, (ii) control (transferred) embryos, and (iii) tetraploid (transferred) embryos. By regressing the log cell number against time, it was possible to estimate the gradient of each line using least squares. The regression was weighted by the sample size for each mean value. From the gradient it was possible to estimate the average cell doubling time for each series. No significant difference was found between the cell doubling times of control series (ii) and the tetraploid series (iii), but the cell doubling times between control series (i) and (ii) were significantly different (see text for details).

Figure 5.2



(iii) CELL DOUBLING TIME OF ELECTROFUSION-INDUCED TETRAPLOID EMBRYOS ISOLATED FROM PSEUDOPREGNANT RECIPIENTS AT VARIOUS TIMES AFTER THE HCG INJECTION FOR INDUCING OVULATION

This group was in all respects identical to control series (ii) except that the embryos were exposed to the electrofusion stimulus during their brief period of incubation *in vitro*. As in series (i) and (ii) above, the mean total cell counts of the tetraploids was established at intervals after the original hCG injection (see Table 5.1). The values obtained were then converted into natural logarithms and plotted graphically against time after the hCG injection. The regression line obtained allowed a cell doubling time (in hours) to be determined, in this series between 74-116 hours after the hCG injection (see Figure 5.2). In this series, the slope of the regression line was not found to be significantly different from the value obtained from control series (ii) above, with a mean cell doubling time ( $\pm$  SEM) of  $14.87 \pm 0.54$  hours. This value was, however, significantly different from the calculated mean doubling time for the embryos in the *in vivo* series. Representative air-dried preparations of tetraploid embryos are shown in Figure 5.3.

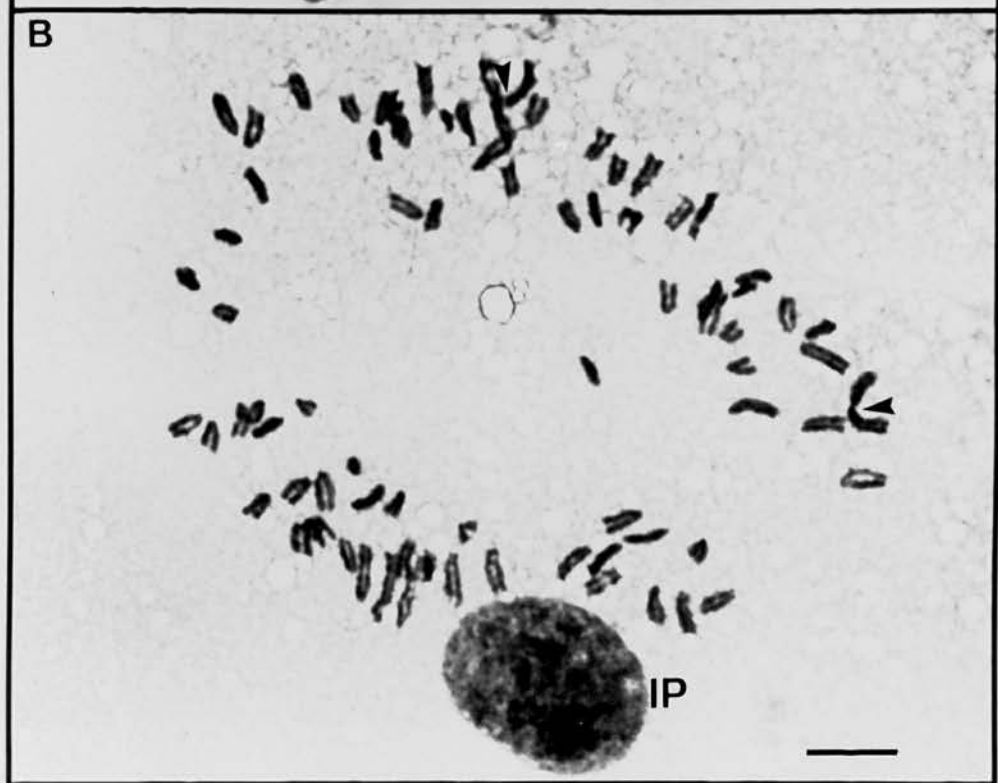
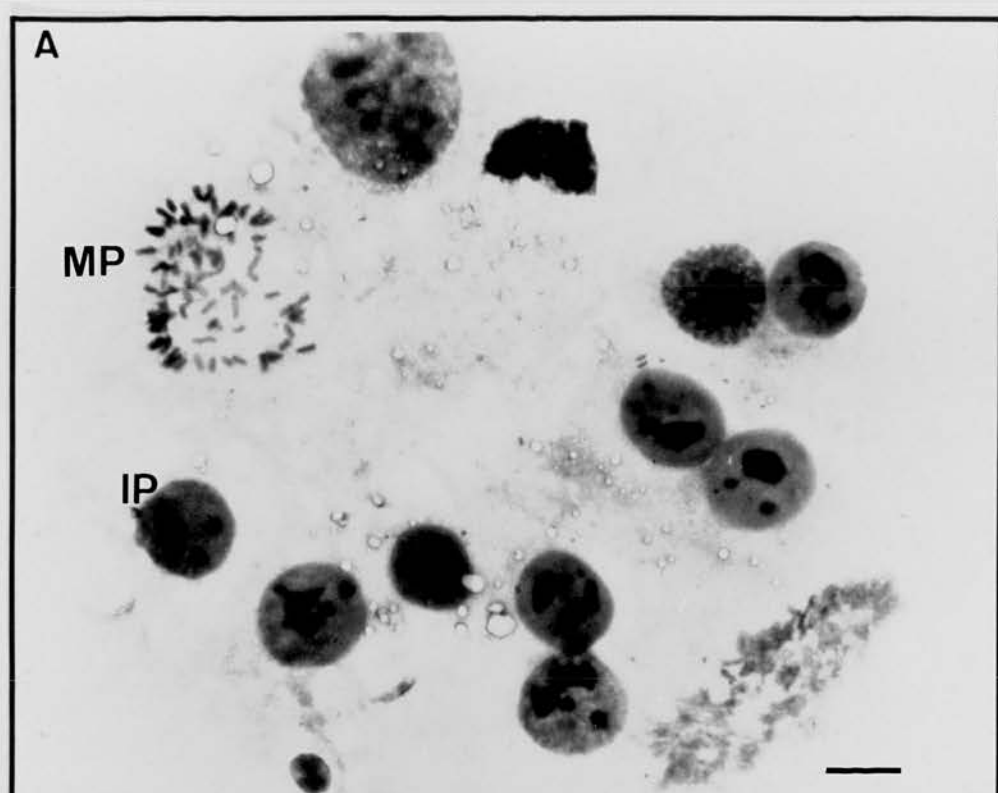
5.3.2 THE LOCATION AND MORPHOLOGICAL APPEARANCE OF CONTROL AND TETRAPLOID EMBRYOS ISOLATED AT VARIOUS TIMES AFTER THEIR TRANSFER TO PSEUDOPREGNANT RECIPIENTS.

In both the control series (ii) and the tetraploid series (iii), embryos were almost exclusively located in the oviduct at all times up to 100-105 hours after the original hCG injection. At 110 hours in the control series (ii), just over 70% of the embryos were still located in the oviduct, while the rest were recovered from the uterus. At 122 hours, however, all of the embryos recovered were located in the uterus. In the case of the tetraploids, at 116 hours after the hCG injection, over 90% of the healthy embryos recovered were located in the uterus.

In the control series, all of the healthy embryos isolated at 70 hours after the hCG injection were compacted morulae (mean cell number  $\pm$  SEM:  $9.28 \pm 0.49$ ). The first blastocysts were

**Figure 5.3**

- A) A representative air-dried preparation of a tetraploid embryo. This embryo consists of 13 cells (10 interphase nuclei (IP), and 3 metaphase plates (MP) one of which is very condensed). (scale bar = 10 $\mu$ m)
- B) A high-power view of a tetraploid metaphase plate consisting of 78 chromosomes, two of which are large "marker" chromosomes (arrow heads). The presence of the two "marker" chromosomes provides unequivocal evidence of the homozygous status of this tetraploid embryo. (scale bar = 10 $\mu$ m)



recovered at 95 hours after hCG , and constituted about 12% of the embryos recovered at this time. By 100 hours, the blastocysts now constituted 74% of the embryos recovered. At 110 hours, all the embryos recovered were at the blastocyst stage, and of these 31% were zona-free, while at 122 hours, 94% of the blastocysts recovered were zona-free.

The situation with regard to the tetraploids was found to be remarkably similar, in that compacting embryos (albeit at the 4-cell stage) were recovered at 74 hours, and only fully compacted embryos at 81 hours (see Figure 5.1c). By 97 hours, 39% of the embryos recovered were at the blastocyst stage, while at 105 hours, 79% were at the blastocyst stage, and of these 4% were zona-free. However, by 116 hours, 79% of the blastocysts recovered from the uterus were zona-free. Curiously, 3 blastocysts and a morula, out of a total of 51 healthy embryos isolated at this time, were recovered from the oviduct. It is apparent, therefore, from these findings that blastocyst formation and the time of achievement of the zona-free state was almost exactly the same as in the appropriate controls (See Figure 5.4).

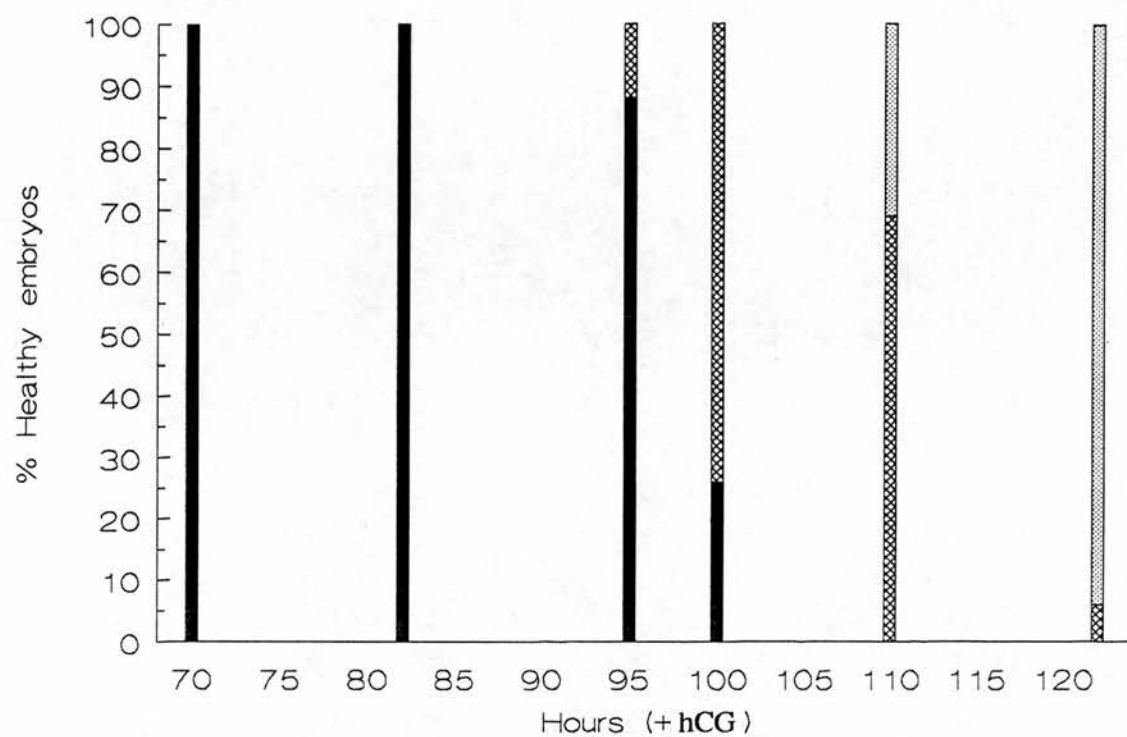


**Figure 5.4**

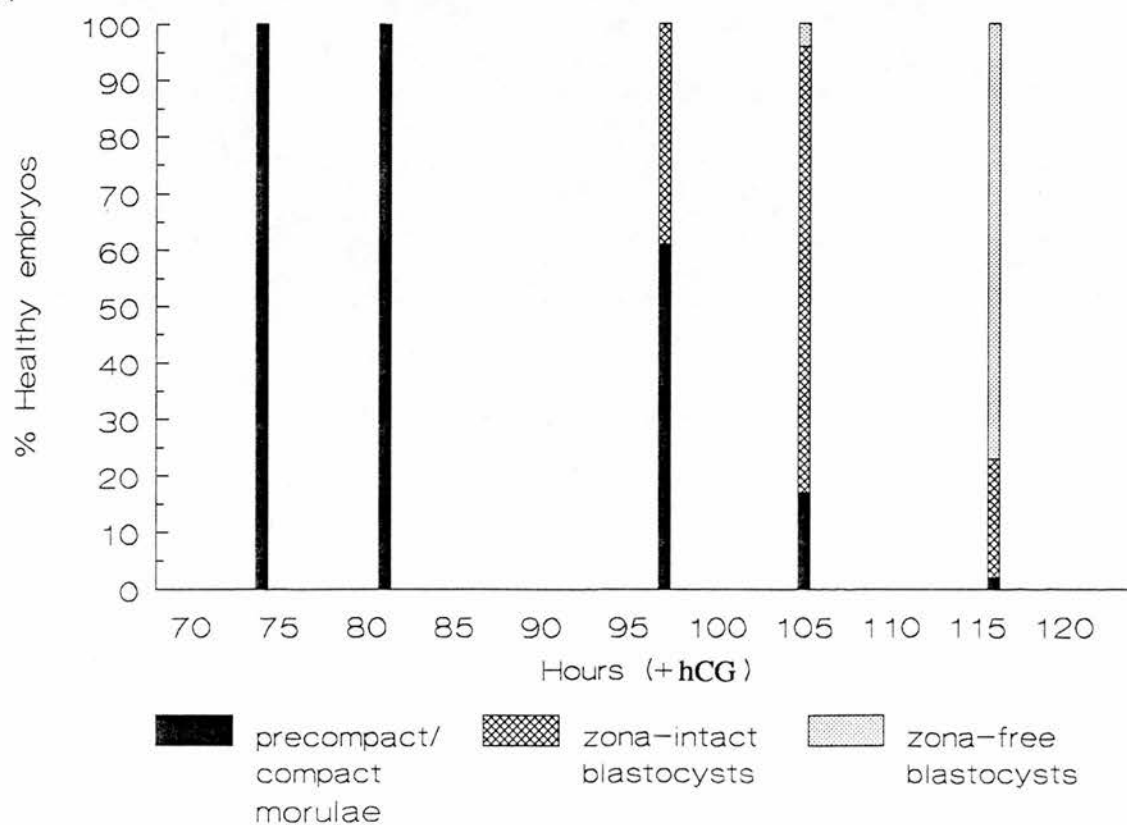
The morphological appearance of (a) diploid (transferred) control embryos, and (b) tetraploid (transferred) embryos isolated at various times after the hCG injection to induce ovulation (morphological appearance based on number of recovered healthy embryos from Table 5.1)

Figure 5.4

(a)



(b)



#### 5.4 DISCUSSION

My results reveal that tetraploid mouse embryos cleave at approximately the same rate during the preimplantation period as diploids. A superficial analysis of cell number at particular stages of preimplantation development can imply this, as others have done. Thus Beatty and Fischberg (1951) found that the mean total cell number of six mouse tetraploids at 3.5 days of gestation was about half that of genetically similar control embryos. Analyses of mouse tetraploid blastocysts by others, also revealed that the tetraploids contained about half the cell number of control blastocysts (Snow, 1975, 1976; Smith and McLaren, 1977; Tarkowski et al., 1977; Spindle, 1981; Kubiak and Tarkowski, 1985).

It is suggested that this reduced cell number in tetraploid embryos results from their suppressed first (or second) cleavage division (or in this study from the induced fusion of their blastomeres), since their *rate* of development appeared to be similar to that of diploids. The tetraploids in this study compacted at the same time as diploids (i.e when they possessed 4 blastomeres, equivalent to about the 8-cell stage in diploid controls, see Figure 5.1c) (see also Kubiak and Tarkowski, 1985; Kato and Tsunoda, 1987), and formed blastocysts at the same time after fertilization as controls (Smith and McLaren, 1977; Eglitis and Wiley, 1981; Petzoldt et al., 1983). It is of interest to note that in my study, fully expanded blastocysts were flushed out from the oviduct at 95-105 hours in both control and tetraploid series. This is clearly due to the fact that these embryos were transferred to asynchronous recipients that were approximately 24 hours earlier in gestation than were the transferred embryos. By 116 hours after the hCG injection virtually all of the control as well as the tetraploid embryos were zona-free and found in the uterus.

It is evident from this study that morphological events such as compaction and blastocyst formation are not dependent on cell number (see also Tarkowski and Wróblewska, 1967; Smith and McLaren, 1977; Surani et al., 1980; Evsikov et al., 1990). It has also been reported that these morphogenetic events are independent of cell-cell interactions (Surani et al., 1980)

and nuclear-cytoplasmic ratio (Evsikov et al., 1990). Popular views are that these morphogenetic events are dependent on the number of DNA cycles of replication (Smith and McLaren, 1977), although Dean and Rossant (1984) have suggested that such events may also be independent of the number of DNA cycles and instead may be controlled by other biological clocks. Since octaploid mouse embryos also divide and cavitate at the same rate as control embryos (Winkel and Nuccitelli, 1989), it would suggest that the mechanisms underlying cell division and blastocyst formation are also independent of an increase in ploidy. These and my previous findings (Henery and Kaufman, 1992b, 1993a) demonstrate that an increase in ploidy does not affect the potential of embryos to achieve the morphological stages of normal preimplantation development.

The tetraploids in this study clearly had a reduced cell number in comparison to controls. However, a reduced cell number appears not to be detrimental to embryonic development. Tarkowski (1959), for example, obtained normal liveborn young following the transfer to appropriate recipients of "half blastocysts" (i.e. embryos where one blastomere at the 2-cell stage was destroyed) (see also, Tsunoda and McLaren, 1983; Rands, 1986b; Biggers and Papaioannou, 1991), and good postimplantation development has recently been obtained from tetraploid mouse blastocysts presumably possessing half the cell number of diploid controls (Kaufman and Webb, 1990; Kaufman, 1991a, 1992a). Even with a reduced inner cell mass (ICM) cell number, which occurs in consequence of a reduction in total blastocyst cell number (Tarkowski and Wróblewska, 1967), embryonic development still appears to proceed normally. No attempt was made here to establish ICM number, but unlike the tetraploids examined by Snow (1973, 1975), all of the tetraploid blastocysts analysed in this study had an obvious ICM. Tarkowski and Wróblewska (1967) demonstrated that even 1/8 blastomeres of a mouse embryo are capable of becoming blastocysts, though the proportion of trophoblastic vesicles also increased at this stage.

Results indicate that tetraploid blastocysts, even with their reduced total cell number, have a similar expectation of developing into the early postimplantation period as diploids, though their more advanced development is restricted. For example, Tarkowski et al. (1977) found that the implantation rate for their cytochalasin B-induced tetraploids was always above 50% up to day 10 of gestation (although their sample was small). O'Neill et al. (1990) found that on day 10 of gestation, 88.2% of transferred Sendai virus "reconstituted" 1-cell stage tetraploids had implanted, and about 50% of those implants contained developing embryos. Similarly, Kaufman and Webb (1990) described an implantation rate of 78.7% at day 10 of gestation for their tetraploid series, of which about 70% contained healthy embryos (see also Kaufman, 1991a, 1992a). This is in contrast to Snow (1975) who found that of the 60% of the tetraploid blastocysts that implanted, only about 19% contained embryos (including dead ones). The recent studies suggest that the majority of tetraploid blastocysts have the potential to cause a decidual reaction, and a large proportion of them must have a viable ICM since embryonic development has been reported. In this study, at 105h post hCG, about 77% healthy transferred tetraploids were recovered and most of these were zona-intact blastocysts. At 116h, the recovery rate of healthy tetraploids was reduced, but the majority of tetraploids that were recovered were zona-free, and this might indicate that some tetraploids were already implanting at this time.

It is apparent, therefore, that embryos with a reduced cell number are capable of postimplantation development and live birth. Embryos with a reduced cell number apparently regulate their development during the early postimplantation period until their size is indistinguishable from normal diploid littermates. Tarkowski (1959) found that by day 11, "half"-embryos had reached the stage and size characteristic of normal ones. Rands (1986b) also found this but reported that there was an indication that by about 13.5 days, the size of "half"-embryos may have fallen back again, but this difference was not statistically significant. What is also interesting is the fact that "double"-embryos, formed from aggregating preimplantation embryos together, also undergo size regulation, but earlier, at around 6 days (Tarkowski, 1963; Mintz, 1971; Buehr and McLaren, 1974; Lewis and Rossant, 1982; Rands,

1986a). It is clear that the embryo is able to cope with perturbations of cell number. It is possible, however, that while the system may also be sufficiently flexible to accommodate the reduced cell number of the tetraploid embryos during the early postimplantation period, secondary detrimental effects, for example, in relation to critical spatial relationships, may manifest themselves at later stages of gestation which might lead to abnormal development and even death of these embryos.

It is also clearly evident from my results that the experimental handling of embryos has a detrimental effect on their subsequent cleavage rate. The *in vivo* data here, reporting a preimplantation embryonic cell doubling time of approximately 10 hours, are in close agreement with previous studies (Bowman and McLaren, 1970; Allen and McLaren, 1971; Barlow et al., 1972; Streffer et al., 1980; Harlow and Quinn, 1982; Molls et al., 1983). Estimates for cell doubling time for *in vitro* studies are more varied, possibly because of variability in the culture conditions employed. Bowman and McLaren (1970), for example, gave an *in vitro* embryonic cell doubling time of about 24 hours, Harlow and Quinn (1982) 17 hours, while Streffer et al. (1980) gave 12 hours. It is not justified to make an exact comparison between my findings and these *in vitro* studies since the embryos in my study were not cultured exclusively *in vitro*. However, it is interesting to note that the cell cycle time of 2-cell stage embryos that had been isolated and subsequently transferred after a brief incubation period in culture to pseudopregnant recipients, was approximately halfway between the calculated mean doubling time *in vivo* and the published figures for cell doubling time *in vitro*. Furthermore, my findings do not appear to show that the cell cycle time in the tetraploids varied during the period studied. Indeed, it was almost identical in all respects to that of the matched controls. This latter group, therefore, would seem to be a more meaningful control for the type of study undertaken here, than comparing the cell cycle time of the tetraploids with that of the controls *in vivo*.

Although the initial slowing of cleavage appears to occur immediately after the transfer procedure, and indeed is to be expected, the most obvious effect of isolating embryos and briefly incubating them *in vitro* is on their subsequent cleavage rate, which appears to be detrimentally influenced by even the minimal handling of this material. However, there appears to be no apparent long-term effect on subsequent embryonic viability in the case of controls, and even in the case of the tetraploids high rates of implantation and early postimplantation embryonic development are the norm. More advanced development, however, is restricted, presumably because of genetic problems, rather than as a consequence of the initial handling of this material. Kaufman et al. (1989b), for example, reported an implantation rate of 94.4% on day 10 of gestation of normal control embryos that had been transferred at the 1-cell stage, which is an indication of the efficiency of such a procedure. It has been reported that embryo transfer does, however, result in smaller litters and larger, more variable animals after birth (Biggers and Papaioannou, 1991; Willadsen et al., 1991; Walker et al., 1992), but subsequently the growth rate of these animals is regulated to that of normal littermates.

My results demonstrate that under matched experimental conditions, tetraploid mouse embryos develop during the preimplantation period at the same rate as diploids despite their reduced cell number, and that this reduction in cell number is a consequence of the experimental induction of tetraploidy. With the proviso that strain differences and experimental techniques may influence embryonic development, my findings would seem to suggest that the hypothesis proposed by Tarkowski et al. (1977) (see also Dyban and Baranov, 1987), namely that the developmental retardation in the tetraploid embryos primarily results from their reduced proliferative activity, is not applicable at least in relation to their preimplantation development.



## **CHAPTER 6**

**THE RELATIONSHIP BETWEEN CELLULAR AND NUCLEAR VOLUME AND PLOIDY  
IN NUCLEATED RED BLOOD CELLS OF DEVELOPMENTALLY MATCHED DIPLOID  
AND POLYPLOID MOUSE EMBRYOS.**

## 6.1 INTRODUCTION

A relationship between cell and nuclear volume and ploidy has been proposed in relation to polyploids of *recent* origin. In general, an increase in ploidy is associated with an increase in cell size, though such a relationship is not commonly observed in long established polyploid species, where the cellular and nuclear volume tends to decrease towards that characteristic of the diploid parental species (for review, see Beatty, 1957). Such a relationship exists in plant, fish and amphibian polyploids, but this relationship has never been fully explored in mammalian polyploids, even though such an alteration in cell size may induce a geometric imbalance in the embryo (i.e. the number of cells in the embryo may depart from normal to compensate for an alteration in cell size) which may explain the abnormalities and premature death observed in these embryos.

My own study, therefore, is concerned with attempting to quantify whether a defined relationship exists between cell size and ploidy in developmentally matched groups of normal diploid embryos and polyploid mouse embryos. I have quantified cell and nuclear volume using nucleated yolk sac-derived primitive red blood cells in the spontaneous digynic triploid mouse embryos of the LT/Sv strain of mice, in experimentally-produced diandric triploid embryos, and also in homozygous tetraploid mouse embryos produced by electrofusion. The cells of these polyploid embryos have been compared with cells from developmentally matched diploid controls. In the case of the LT/Sv triploid embryos, the developmentally matched controls were of the same genetic background and derived from the diploid littermates of triploid embryos. The primitive red blood cells that were analysed first enter the embryonic vasculature on day 7.5-8 of gestation and diminish in number during days 14 and 15 (Rugh, 1968; Kaufman, 1992b). These cells, as well as their nuclei, have a characteristic spherical shape throughout their existence in the embryonic circulation, which clearly simplifies any morphometrical analysis. I show that a clear relationship does indeed exist between cell size and ploidy in these cells and that such a relationship is only seen in nucleated red blood cells when material from carefully developmentally matched embryonic stages are compared.

## 6.2 MATERIALS AND METHODS

### LT/SV SERIES: DIGYNIC TRIPLOID SERIES AND DIPLOID CONTROL SERIES I

8- to 12-week-old randomly cycling female LT/Sv mice were caged with F1 hybrid males. LT/Sv female mice ovulate both primary and secondary oocytes, all of which are capable of being fertilized (O'Neill and Kaufman, 1987b). Fertilization of primary oocytes results in the production of digynic triploid conceptuses, while their diploid littermates result from the fertilization of normal secondary oocytes. Early each morning the females were checked for the presence of a vaginal plug, and the latter was taken as evidence of mating. The morning of finding a vaginal plug was considered to be the first day of gestation.

### DIANDRIC TRIPLOID SERIES

8- to 12-week-old female F1 hybrid mice were superovulated and then caged individually with homozygous Rb(1.3)1Bnr male mice. Early pronucleate-stage fertilized eggs were isolated early in the morning on the day of finding a vaginal plug and diandric triploid embryos were produced by standard micromanipulatory procedures, as described previously. These diandric triploid tripronucleate embryos were then transferred unilaterally to the oviducts of recipients on the first day of pseudopregnancy. The day of the transfer was considered to be the first day of gestation.

### TETRAPLOID SERIES

F1 hybrid females were superovulated and mated to Rb(1.3)1Bnr male mice. The females were killed early on the morning of the second day of pregnancy, and 2-cell embryos isolated. These embryos were subjected to the electrofusion stimulus to fuse their two blastomeres together to form tetraploids, as described previously. These 1-cell (tetraploid) embryos were then transferred unilaterally to the oviducts of pseudopregnant recipients.

## DIPLOID CONTROL SERIES II

8- to 12-week old randomly cycling female F1 hybrid mice were caged with F1 hybrid males.

The morning of finding a vaginal plug was considered to be the first day of gestation.

## ANALYSIS OF POSTIMPLANTATION STAGES OF DEVELOPMENT

The females from the control and tetraploid series were autopsied between the 8th and 15th days of gestation, while the females from the digynic and diandric triploid series were autopsied between 8th and 10th day of gestation. The decidual swellings were isolated from the uterine horns and the embryos were isolated from within their extra-embryonic membranes and then fixed in Bouin's fixative for subsequent histological examination. Digynic triploid embryos are easily distinguished from their diploid littermates by their abnormal and retarded development and small size. However, the extra-embryonic membranes of the embryos were used to confirm their chromosome constitution, using a modification of the technique described by Evans et al. (1972). It was assumed that all of the controls had a normal diploid chromosome constitution.

The embryos fixed for histological examination were serially-sectioned in the transverse plane, cut at a nominal thickness of 7 $\mu$ m (embryos up to 11 days post coitum (p.c.)), and at 8 $\mu$ m for developmentally more advanced embryos, and subsequently stained with haematoxylin and eosin. The exact developmental age of the triploid and tetraploid embryos was established by comparing their postcranial morphological/histological features with those of diploid control embryos in M.H. Kaufman's reference collection of serially-sectioned mouse embryos, based on the morphological staging schemes of Theiler (1989) and Kaufman (1990). The cranial features of the tetraploid embryos were not compared, or included in the analysis, as these were generally grossly abnormal (see Kaufman and Webb, 1990), whereas the histological features of the postcranial viscera were often normal. Since the developmental period that the red blood cells of the triploid embryos could be analysed in was so short, it was more suitable to group both the triploid and control diploid embryos into developmental periods of half-days. This increased the sample size for each point studied.

Mean cellular and nuclear volume was established in nucleated (primitive) red blood cells based on their cellular and nuclear diameters as described previously. These cells were chosen because of the spherical or near spherical nature of their nuclei and their near spherical cellular outline. The average cellular and nuclear diameters (+ standard deviation, S.D.) of the nucleated red blood cells of control diploid, triploid and tetraploid embryos were:

|                   | mean cell diameter (µm)<br>(+ S.D.) | mean nuclear diameter (µm)<br>(+ S.D.) |
|-------------------|-------------------------------------|--|
| Control diploid   | 9.5 (0.68)                          | 5.7 (1.00)                             |
| Diandric triploid | 10.6 (0.67)                         | 7.2 (0.48)                             |
| Digynic triploid  | 11.1 (0.43)                         | 7.2 (0.28)                             |
| Tetraploid        | 11.4 (0.90)                         | 6.7 (1.24)                             |

### 6.3 RESULTS

It was assumed that the triploid and tetraploid embryos were healthy and appropriate for analysis if on recovery their hearts were undergoing vigorous spontaneous contractions, and there was no evidence of vascular stasis within the cephalic region (a common feature in dying embryos), otherwise they were not processed for histology. It was usual for digynic but not diandric triploid embryos to display various craniofacial and often cardiac abnormalities, but this did not exclude them from analysis if they were apparently healthy based on the criteria indicated above.

#### 6.3.1 COMPARISON OF THE CELLULAR AND NUCLEAR VOLUME OF THE PRIMITIVE RED BLOOD CELLS OF TRIPLOID AND CONTROL DIPLOID EMBRYOS

The cellular and nuclear volumes of primitive red blood cells in a total of 16 digynic triploid and 22 diandric and 30 developmentally matched control diploid embryos (including 7 LT/Sv diploid embryos) were determined from an analysis of histological serial-sections of these embryos. The analysis was carried out between 8 - 10 days p.c. in the case of the triploid embryos, and 8 - 10.5 days p.c. in the case of diploid control embryos.

The cellular and nuclear volumes of the primitive red blood cells of LT/Sv control diploid embryos were compared with those from diploid embryos of a similar developmental age from control series II. The LT/Sv diploid embryos provide an important inbuilt control for the digynic triploids in that they have been subjected to an identical maternal environment prior to their isolation. Since there was no statistical difference between the measurements obtained from the two groups of controls, their data were pooled in order to increase the sample size.

(i) COMPARISON BETWEEN VALUES OBTAINED FROM THE ANALYSIS OF  
DIANDRIC TRIPLOID AND DIGYNIC TRIPLOID EMBRYOS

The mean cell and nuclear volumes of the primitive red blood cells of diandric and digynic triploid embryos were determined from an analysis of approximately 100 cells per embryo. These values were plotted and regression lines drawn. No statistical difference in cellular or nuclear volume was observed between the diandric and digynic triploid embryos ( $p > 0.05$ ) (Table 6.1). From this finding, I infer that the cellular and nuclear volumes of primitive red blood cells of spontaneously occurring digynic triploid and experimentally-produced diandric triploid embryos show the same characteristic response, namely a predictable increase in volume, to an increase in ploidy compared to values observed in diploid control embryos.

(ii) THE CELLULAR VOLUME OF THE PRIMITIVE RED BLOOD CELLS IN  
TRIPLOID AND CONTROL DIPLOID EMBRYOS

The volumes of the primitive red blood cells were determined from an analysis of approximately 100 cells per embryo. The mean cell volumes for the triploid and diploid embryos were plotted and lines of regression drawn (Figure 6.1a). The volumes of these two groups were found to be statistically different ( $p < 0.05$ ). A ratio of triploid : diploid cell volume was determined for each of the time points studied (Table 6.2). The average of the values of triploid : diploid cell volume ratios was approximately 1.5:1. This is as expected if it is assumed that there is a direct relationship between cellular volume and ploidy, with triploid cells having a cellular volume 1.5x that of diploids.

(iii) THE NUCLEAR VOLUME OF PRIMITIVE RED BLOOD CELLS IN TRIPLOID  
AND CONTROL DIPLOID EMBRYOS

Corresponding nuclear volume values were obtained at the same time as cellular volumes for the triploid and two groups (pooled) of diploid embryos. The mean nuclear volumes obtained over the period of study for the triploid group and the diploid embryos were plotted, and



**Table 6.1** Cellular and nuclear volume of diandric triploid and digynic triploid primitive red blood cells measured in developmentally matched material between 8-10 days p.c.

| Developmental stage analysed (days p.c.) | DIANDRIC TRIPLOIDS   |                    |   |  | DIGYNIC TRIPLOIDS    |                    |   |  |
|--|----------------------|--------------------|---|--|----------------------|--------------------|---|--|
|  | No. embryos analysed | No. cells analysed | Mean cell volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) | Mean nuclear volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) | No. embryos analysed | No. cells analysed | Mean cell volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) | Mean nuclear volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) |
| 8-8.5                                    | 4                    | 300                | 540.1<br>( $\pm 82.0$ )                             | 167.6<br>( $\pm 28.6$ )                                | -                    | -                  | -   | -  |
| 8.5-9                                    | 4                    | 400                | 682.6<br>( $\pm 32.3$ )                             | 203.1<br>( $\pm 23.0$ )                                | 4                    | 317                | 688.4<br>( $\pm 21.5$ )                             | 174.6<br>( $\pm 10.8$ )                                |
| 9-9.5                                    | 13                   | 1250               | 614.9<br>( $\pm 21.4$ )                             | 168.1<br>( $\pm 5.8$ )                                 | 6                    | 600                | 713.9<br>( $\pm 37.4$ )                             | 174.5<br>( $\pm 6.1$ )                                 |
| 9.5-10                                   | 1                    | 100                | 628.9*  | 152.6*   | 6                    | 531                | 632.4<br>( $\pm 19.5$ )                             | 140.1<br>( $\pm 3.2$ )                                 |

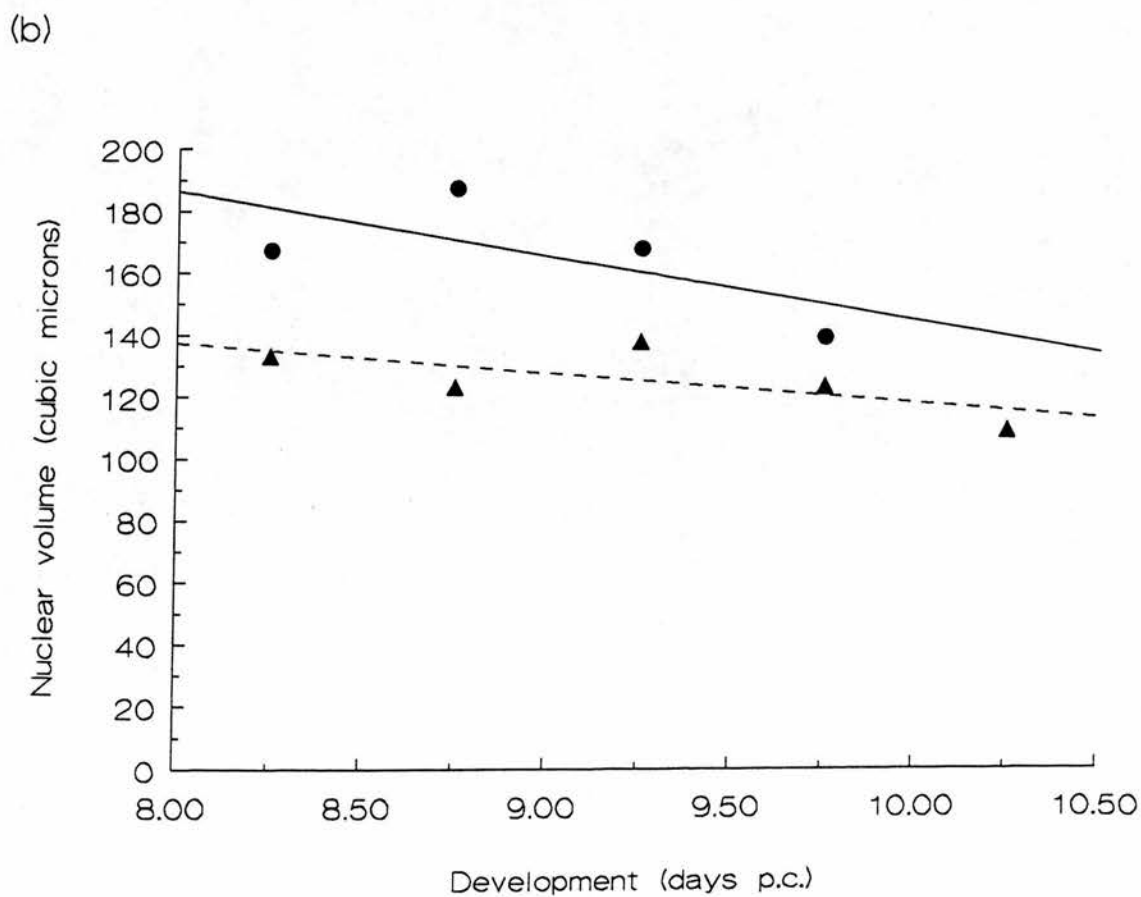
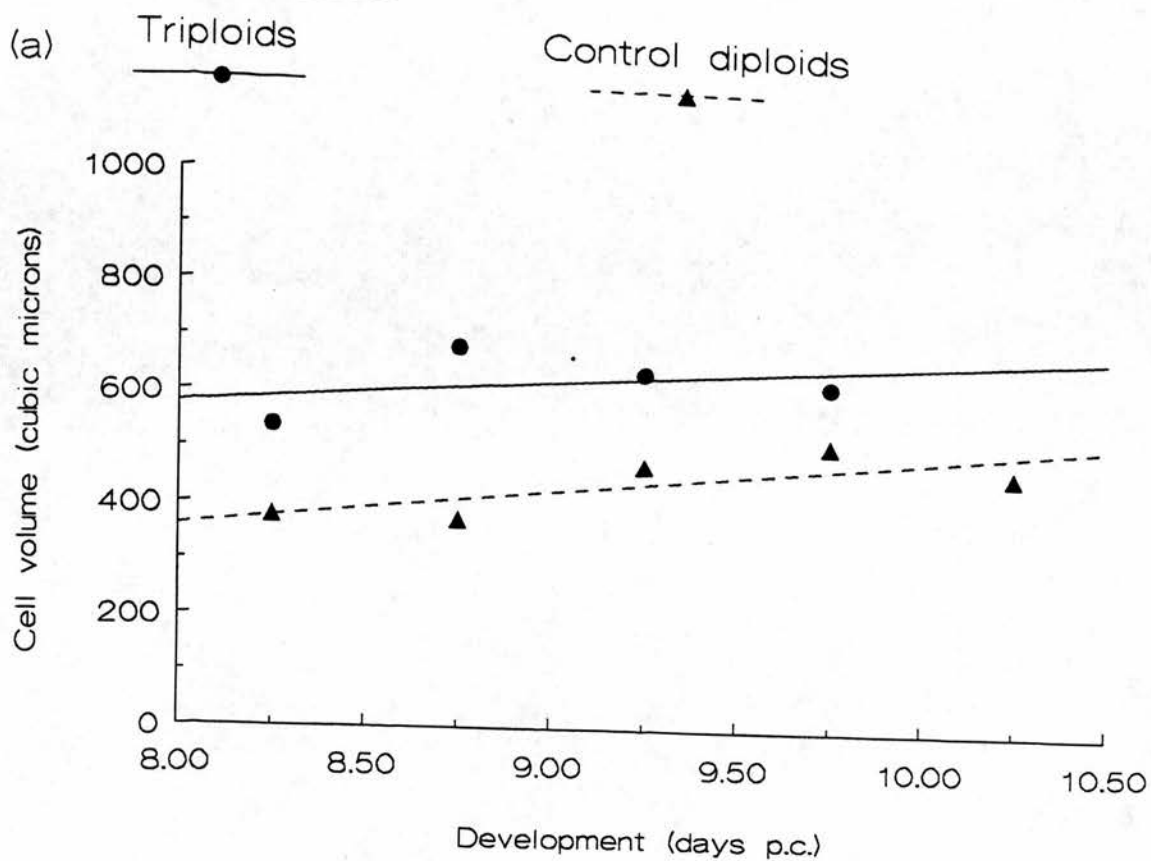
\* No SEM as only one embryo measured  
There was no statistical difference between the values for the cellular and nuclear volume in the primitive red blood cells of diandric and digynic triploid embryos ( $p > 0.05$ ).

Figure 6.1

- a) Regression lines obtained when raw mean values for overall cellular volume obtained from the analysis of control diploid and developmentally matched triploid (diandric and digynic) primitive nucleated red blood cells are plotted against developmental age of embryos. The control and triploid regression curves are significantly different ( $p < 0.05$ ).
- b) Regression lines obtained when raw mean values for primitive red blood cell nuclear volumes of control diploid and developmentally matched triploid (diandric and digynic) embryos are plotted against developmental age of embryos. The control and triploid regression line values are significantly different ( $p < 0.05$ ).

Since the confidence limits of these regression lines were found to overlap, they are of little predictive value statistically speaking, and therefore have been omitted. The regression lines are, however, valuable since they show clearly the general difference in size of the diploid and triploid cell and nuclear volume values.

Figure 6.1





regression lines drawn (Figure 6.1b). There was a statistical difference observed between the nuclear volumes of the two groups ( $p < 0.05$ ). The average ratio of triploid : diploid nuclear volume was about 1.3:1 ( Table 6.2).

### 6.3.2 COMPARISON OF THE CELLULAR AND NUCLEAR VOLUME OF THE PRIMITIVE RED BLOOD CELLS OF TETRAPLOID AND CONTROL DIPLOID EMBRYOS

In this study, the primitive red blood cell and nuclear volume of a total of 24 tetraploid and 39 developmentally matched control diploid embryos were analysed in the developmental period between 8.25-14.5 days p.c..

#### (i) THE CELLULAR VOLUME IN THE PRIMITIVE RED BLOOD CELLS OF TETRAPLOID AND DIPLOID EMBRYOS

The mean value for the overall cellular volume of tetraploid and control diploid primitive red blood cells was established by measuring approximately 100 cells from each of a selection of serially-sectioned embryos at each time point studied (see Table 6.3). The ratio of tetraploid data to control data was found to be close to the predicted theoretical ratio of 2:1. The values for controls were found to be significantly different from tetraploid values ( $p < 0.01$ ). The large variation in individual values for cell volume necessitated converting them to natural logarithms as a means of providing data for which a reasonable curve could be produced. These values were plotted and a 5th order polynomial curve was fitted using weighted least squares. The preparation of a curve by this means was chosen as it provided the best fit for the data. The latter was essential if the curve obtained from the results was to have any predictive value. 95% confidence limits for the curve were calculated and also drawn. Both control and tetraploid regression curves show an early increase in cell volume, reaching a maximum at approximately 10.5-11.5 days p.c., then a gradual decrease in cell volume until they eventually disappear from the circulation (see Figure 6.2a).

**Table 6.3 Cellular and nuclear volumes of control diploid and tetraploid primitive red blood cells measured in developmentally matched material between 8.25-14.5 days p.c..**

There was a statistical difference between the values for the cellular and nuclear volumes of the primitive red blood cells in tetraploid and diploid control embryos ( $p < 0.01$ ) for each of the time points studied.

# CONTROLS

# TETRAPLOIDS

| Developmental stage (days p.c.) | No. embryos analysed | No. cells analysed | Mean cell volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) | Mean nuclear volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) | No. embryos analysed | No. cells analysed | Mean cell volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) | Mean nuclear volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) | 4N:2N cell volume | 4N:2N nuclear volume |
|---------------------------------|----------------------|--------------------|---|--|----------------------|--------------------|---|--|-------------------|----------------------|
| 8.25                            | 0                    | 0                  | 0   | 0  | 1                    | 28                 | 690.4*  | 254.0*   | NA                | NA                   |
| 8.5                             | 2                    | 200                | 353.2 ( $\pm 9.9$ )                                 | 129.8 ( $\pm 14.5$ )                                   | 0                    | 0                  | 0   | 0  | NA                | NA                   |
| 9.0                             | 8                    | 800                | 352.5 ( $\pm 16.5$ )                                | 118.2 ( $\pm 5.3$ )                                    | 3                    | 300                | 657.8 ( $\pm 49.2$ )                                | 202.9 ( $\pm 10.2$ )                                   | 1.87:1            | 1.72:1               |
| 9.5                             | 2                    | 200                | 390.8 ( $\pm 14.3$ )                                | 119.2 ( $\pm 2.8$ )                                    | 3                    | 300                | 675.3 ( $\pm 10.9$ )                                | 216.7 ( $\pm 21.8$ )                                   | 1.73:1            | 1.82:1               |
| 9.75                            | 1                    | 100                | 447.2*  | 130.8*   | 0                    | 0                  | 0   | 0  | NA                | NA                   |
| 10.0                            | 6                    | 600                | 477.6 ( $\pm 31.0$ )                                | 112.0 ( $\pm 6.8$ )                                    | 2                    | 200                | 832.4 ( $\pm 103.1$ )                               | 165.8 ( $\pm 20.0$ )                                   | 1.74:1            | 1.48:1               |
| 10.25                           | 2                    | 200                | 521.0 ( $\pm 11.1$ )                                | 88.3 ( $\pm 5.2$ )                                     | 3                    | 300                | 954.8 ( $\pm 18.6$ )                                | 193.7 ( $\pm 13.3$ )                                   | 1.83:1            | 2.19:1               |
| 10.5                            | 1                    | 100                | 525.4*  | 92.1*  | 1                    | 100                | 1066.8*   | 146.2*   | 2.03:1            | 1.59:1               |
| 11.5                            | 1                    | 100                | 484.9*  | 54.1*  | 1                    | 100                | 1098.7*   | 172.1*   | 2.27:1            | 3.18:1               |
| 12.0                            | 5                    | 500                | 434.0 ( $\pm 19.7$ )                                | 68.6 ( $\pm 2.8$ )                                     | 3                    | 300                | 681.3 ( $\pm 52.2$ )                                | 93.9 ( $\pm 11.7$ )                                    | 1.57:1            | 1.37:1               |
| 12.5                            | 2                    | 200                | 384.0 ( $\pm 19.8$ )                                | 36.6 ( $\pm 4.3$ )                                     | 1                    | 100                | 706.6   | 86.7   | 1.84:1            | 2.36:1               |
| 13.0                            | 2                    | 200                | 371.4 ( $\pm 6.8$ )                                 | 40.9 ( $\pm 2.4$ )                                     | 0                    | 0                  | 0   | 0  | NA                | NA                   |
| 13.5                            | 5                    | 500                | 361.2 ( $\pm 38.0$ )                                | 30.1 ( $\pm 1.3$ )                                     | 3                    | 300                | 557.2 ( $\pm 66.5$ )                                | 56.8 ( $\pm 4.2$ )                                     | 1.54:1            | 1.89:1               |
| 14.5                            | 2                    | 200                | 229.2 ( $\pm 1.5$ )                                 | 24.7 ( $\pm 0.5$ )                                     | 3                    | 300                | 533.1 ( $\pm 13.9$ )                                | 58.2 ( $\pm 5.4$ )                                     | 2.33:1            | 2.36:1               |

\* No SEM as only one embryo analysed.

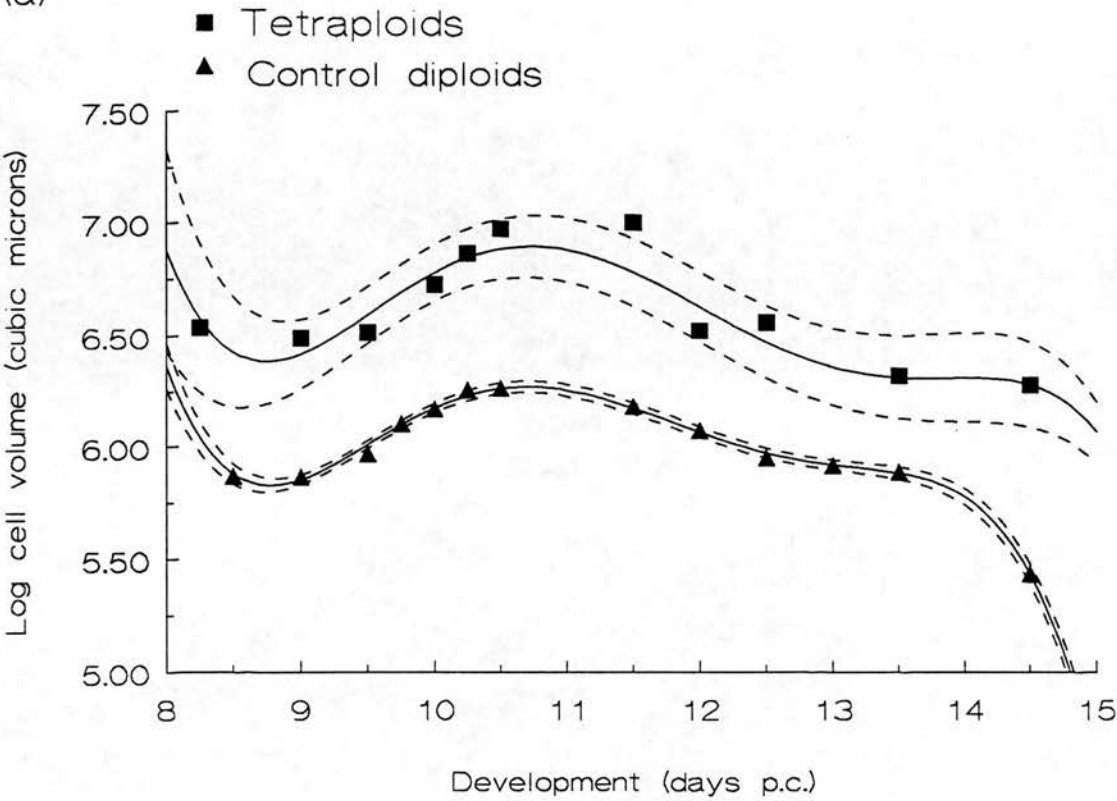


Figure 6.2

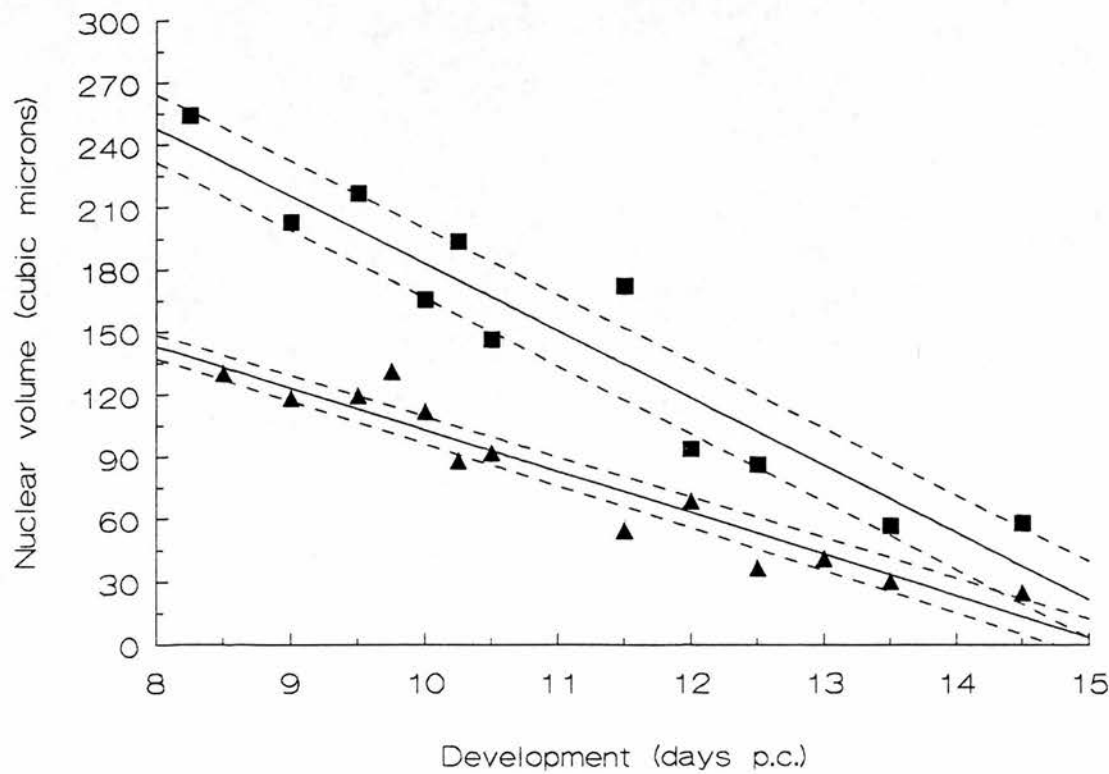
- a) Regression (5th order polynomial) curves obtained when natural logarithms of the mean values for overall cellular volume obtained from the analysis of control diploid and developmentally matched tetraploid primitive nucleated red blood cells are plotted against developmental age of embryos. 95% confidence limits for each curve are also illustrated (dotted lines). The control and tetraploid regression curves are significantly different ( $p < 0.01$ ).
- b) Regression lines obtained when raw mean values for primitive red blood cell nuclear volumes of control diploid and developmentally matched tetraploid embryos are plotted against developmental age of embryos. 95% confidence limits for each line are also illustrated (dotted lines). The control and tetraploid regression line values are significantly different ( $p < 0.01$ ).

Figure 6.2

(a)



(b)



(ii) THE NUCLEAR VOLUME IN THE PRIMITIVE RED BLOOD CELLS OF  
TETRAPLOID AND CONTROL EMBRYOS

Corresponding values for primitive red blood cell nuclear volume for the tetraploid and control diploid embryos described above were also obtained from the mean of approximately 100 cells per embryo (see Table 6.3). These raw values were plotted, regression lines and corresponding 95% confidence limits fitted as indicated above, and the regression lines were found to be significantly different ( $p < 0.01$ ) (see Figure 6.2b). The tetraploid and diploid red blood cells both showed a paralleled (and predictable) decrease in their nuclear volume with increased developmental age. The ratio of tetraploid to control values was again found to be close to the predicted theoretical ratio of 2:1.

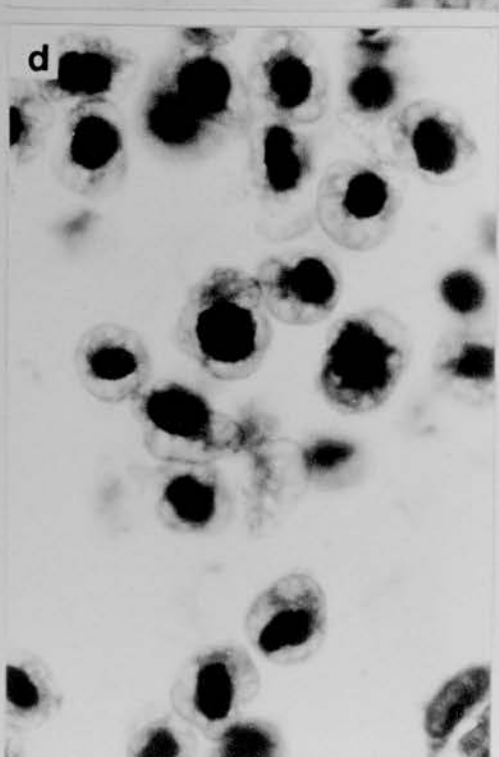
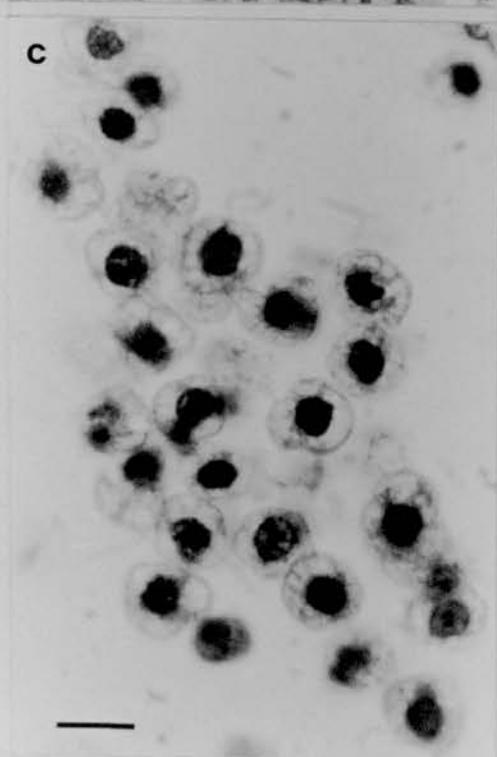
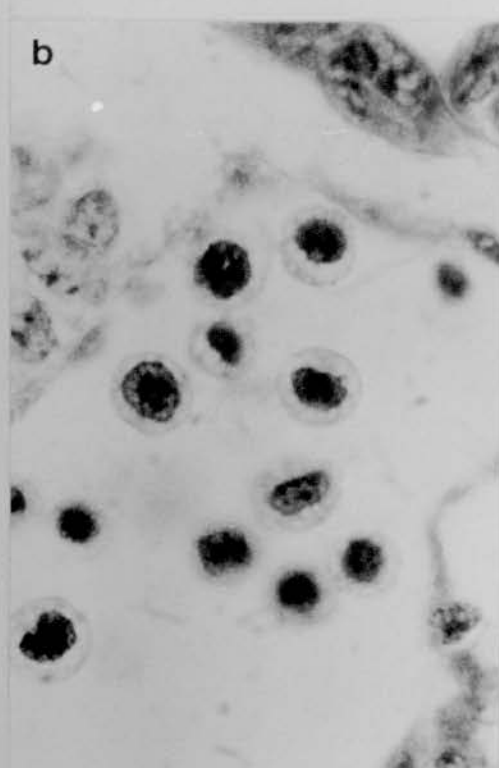
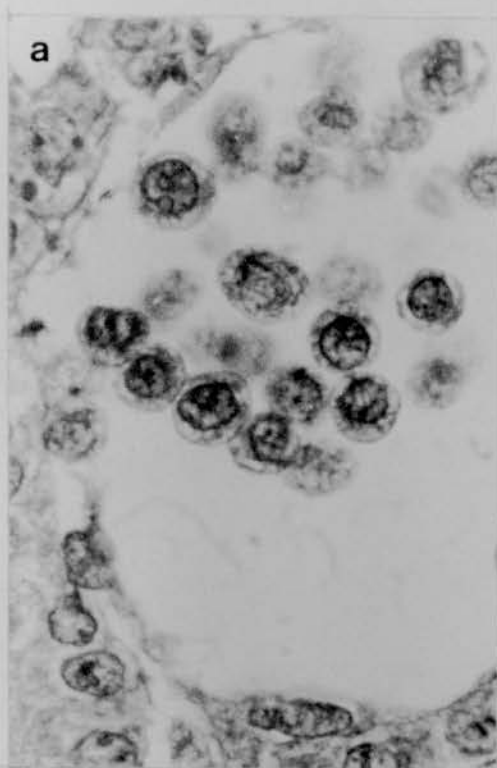
A representative selection of photomicrographs of control and tetraploid nucleated primitive red blood cells from developmentally matched embryos at 9 and 10.5 days p.c. are illustrated in Figure 6.3.

The regression curves and lines and their confidence limits for the red blood cellular and nuclear volumes of tetraploid and control diploid embryos, respectively, fitted over the developmental period from 8.25-14.5 days p.c., are of important predictive value. However, while the triploid values are significantly different from those of the diploid values, statistically speaking, the predictive powers of these regression lines are limited since their confidence limits were found to overlap.

Detailed cytogenetic analyses of the mitoses prepared from the extra-embryonic membranes of triploid, tetraploid and diploid control embryos revealed that all the cells studied, in which accurate chromosome counts could be made, had a normal (ie euploid) chromosome constitution.

**Figure 6.3**

Photomicrographs of histological sections showing comparable regions within the dorsal aortae of control and developmentally matched tetraploid mouse embryos. All sections are stained with haematoxylin and eosin, and are illustrated at the same magnification (X 1000). Scale bar = 10  $\mu\text{m}$ . a and c, control embryos at 9 and 10.5 days p.c., respectively. b and d, tetraploid embryos at 9 and 10.5 days p.c., respectively.



## 6.4 DISCUSSION

A relationship between cell size and ploidy in polyploids of *recent* origin has previously been proposed (Fankhauser, 1945; Fischberg, 1948; and for reviews, see Beatty, 1957; Kaufman, 1983; Dyban and Baranov, 1987) but has never been quantified, as far as I am aware, in any species by detailed morphometric analysis. Earlier work with amphibian embryos, however, established that there was a direct relationship between cell and/or nuclear volume and the number of chromosome sets present.

In the present study, I have observed that both cell and nuclear volume are indeed directly related to ploidy in early postimplantation mouse embryos when *comparable* cells are studied in *developmentally matched* diploid embryos. The cell and nuclear volumes of yolk sac-derived primitive red blood cells of triploid mouse embryos were significantly larger than those of comparable cells in developmentally matched control embryos when analysed between 8-10 days p.c.. In the case of tetraploid mouse embryos, the cell and nuclear volumes of their primitive red blood cells were found to be almost exactly twice those of comparable diploid cells from developmentally matched control diploid embryos when these were analysed between 8.25-14.5 days p.c.. It is important to stress, however, that this relationship is only seen when comparable cells are analysed in developmentally matched material, since the absolute volume of a cell and its nucleus can vary considerably depending on a number of factors, such as its functional activity, the stage in the cell cycle, and at different stages during its lifespan (Watson, 1977).

An important feature of the triploid study was that I was able to analyse primitive red blood cells that had been isolated from digynic triploid and diploid LT/Sv strain littermates from spontaneously cycling LT/Sv strain females previously mated to F1 hybrid males. Clearly these embryos had been subjected to identical conditions in both the pre- and early postimplantation period. The fact that there was no significant difference between the cellular and nuclear volumes of the control values found in the LT/Sv study and in the F1 x F1 study (in

which spontaneously cycling females were used) suggests that the difference in the genetic background of the embryos studied had no significant effect on the cellular and nuclear volumes of their primitive red blood cells. More importantly, the fact that I have demonstrated that there was no significant difference between the cellular and nuclear volumes of the nucleated red blood cells of diandric and digynic triploid embryos despite their genetic dissimilarity, suggests that the relationship between ploidy and volume is a predictable one if the tissues analysed are from carefully developmentally matched embryos. It has been suggested that superovulation, *in vitro* culture and micromanipulation (as occurred in the diandric triploid group), each have a long term detrimental effect on embryonic development, resulting in increased birth weights and prolonged gestation (Biggers and Papaioannou, 1991; Willadsen et al., 1991; Walker et al., 1992). While such findings may exist at birth, my own findings seem to suggest that during the early postimplantation period such differences arising from these techniques are negligible, if at all apparent (Henery and Kaufman, 1992b).

A relationship between cell volume and ploidy is a well known phenomenon in amphibians (Fankhauser, 1941, 1945; Fischberg, 1948), plants (Winkler, 1916; Dawson, 1962) and fish (Swarup, 1959; Small and Benfrey, 1987). That such an increase in cell size does occur in mammalian polyploids has previously been observed in relation to the definitive (i.e. non-nucleated) red blood cells of human triploid fetuses (Fisk et al., 1989) and in human triploid neonates (Pai et al., 1982; Sadowitz et al., 1984). In rabbits, Bomsel-Helmreich (1971) reported an increase in size in the definitive red blood cells of triploid embryos. Similarly, Snow (1973, 1975) noted that cell size in tetraploid mouse embryos was increased, and it was suggested that nucleated yolk sac-derived blood cells in these embryos were about four times the volume of corresponding diploid cells. Such an increase in cell size has also been reported to occur in human diploid-polyploid mosaic tissues (Kohn et al., 1967).

These observations give support to the proposal that in *recent* polyploids, cell size is in some way related to ploidy. However, as far as I am aware, this is the first detailed study in which morphometric means have been applied to investigate this phenomenon. The findings from my



study will be of importance in analysing the underlying basis of the morphological abnormalities that undoubtedly occur in polyploids. Such an increase in cell volume, for example, will ultimately decrease the surface area to volume ratio which might become a limiting factor for cellular metabolism in polyploids (Szarski, 1976). In recent polyploids, an increase in cell size is also related to a concomitant decrease in cell number, so that the polyploid organism is of approximately similar proportions to a diploid organism (Beatty and Fischberg, 1951; Beatty, 1957). In triploid and tetraploid mouse embryos, body size is invariably smaller than that of developmentally comparable diploid embryos (Beatty and Fischberg, 1951; Surani and Barton, 1983; Kaufman and Speirs, 1987; Kaufman et al., 1989b, c; Kaufman and Webb, 1990; Kaufman, 1991a). This study has shown that cell size, at least that of the primitive red blood cells, is increased in these embryos, which must necessarily imply that the cells in the tissues are more densely packed, or that there is likely to be an overall reduction in the total cell population of the embryo. This decrease in cell number does not occur, however, during the preimplantation development of these embryos (Henery and Kaufman, 1991, 1992b, 1993a). The possibility exists that triploid embryos may undergo size regulation in their early postimplantation development, as it does when cell number is increased in, for example, aggregation chimeric embryos (Tarkowski, 1963; Buehr and McLaren, 1974; Mintz, 1971; Lewis and Rossant, 1982; Rands, 1986a). This may involve a slowing of the cell cleavage rate of triploid cells during this time to induce a reduction in total cell number. Alternatively, programmed cell death may account for a reduced cell number. Such a reduction will prevent the embryo from being of giant proportions. It would be instructive, therefore, to carry out a series of morphometric analyses of cell number in the tissues of early postimplantation triploid embryos and subsequent stages, to determine if such a reduction in cell number exists and when it occurs.

It is an important observation in this study, that while the cell and nuclear volume of the primitive red blood cells of diandric and digynic triploid mouse embryos were similar, the morphological features of these embryos are quite different. The diandric triploids appear to be morphologically normal up to the forelimb bud stage when they possess about 25-30 pairs of

somites, while the digynic triploids invariably exhibit craniofacial abnormalities which may be associated with axial neural tube defects, and in some embryos, cardiovascular abnormalities may also be present (Kaufman and Speirs, 1987; Kaufman et al., 1989b, c; Kaufman, 1991b). The morphological abnormalities observed in digynic triploids cannot, therefore, be a result of an increase in cell size or a reduction in cell number, since the diandric triploids encounter the same geometric problems, although both types of triploid embryos die at the same stage in development. While there is clearly a close relationship between cell volume and ploidy in diandric and digynic triploid mouse embryos, it will be interesting to investigate whether there is a quantitative and predictable relationship between gene products and ploidy as predicted by Epstein (1986). It is likely that the morphological differences observed between diandric and digynic triploids is related to the phenomenon of genomic imprinting.

In the case of tetraploids, their cell number at the blastocyst stage, and presumably at the time of implantation, is approximately half that of diploid embryos (although, their *rate* of development is similar to diploids). Since their cells also show a predictable relationship with ploidy and are almost twice that of the diploid cell size, such a slowing of the cleavage rate in the early postimplantation period need not occur. Whether such regulatory mechanisms do operate could be examined in tetraploid embryos produced by pronuclear manipulation. Thus these embryos would initiate development with the same number of cells as diploid embryos. A resulting embryo of normal proportions would indicate that size regulation mechanisms operate not only for a perturbation in cell number but also for a perturbation in cell size.

This relationship between cell size and ploidy will also be of diagnostic importance in those situations where it is necessary to analyse histologically the cellular events that occur in the intact implantation site. Clearly, in these instances, it would be technically impossible at the same time to analyse the chromosome constitution of the embryos by direct cytogenetic means. My results are, therefore, plotted graphically in order to facilitate this exercise. During the developmental period of diploid controls studied, namely between 8-14.5 days p.c., red blood cellular and nuclear volume changes in a predictable fashion. After day 11, the primitive

nucleated red blood cell population is gradually replaced by the definitive erythrocytes which are derived initially from the liver, and later from the spleen and bone marrow. During the overlap period between 11-15 days p.c., however, the primitive erythrocytes play a diminishingly important role, and this coincides with their eventual death and removal from the circulation.

It is unclear why the nuclear and cellular volumes of diploid, triploid and tetraploid primitive red blood cells varied to the extent that was observed here. While I cannot exclude the possibility that some of these cells may have lost one or more chromosomes during previous divisions, and could account for some small variability in their volume, this possibility seems unlikely, as the cytogenetic analyses of extra-embryonic tissues revealed that these embryos always had a normal (i.e. euploid) chromosome constitution. While clearly some of the cells analysed might have been aneuploid, it was hoped that the analysis of usually between 100 to 600 cells per group would increase the accuracy of the mean values reported for each point studied. The fact that, once differentiated, these cells do not divide again, would seem to exclude the possibility that the variation in size seen might be accounted for by the analysis of cells at different stages during the cell cycle.

This study, I believe, confirms that, like plants, fish and amphibians, mammals also show a predictable relationship between cell size and ploidy. Such a relationship requires that the tissues of these embryos be modified to account for this increase in size of its component cells or that the number of component cells in the polyploid embryo be reduced if the embryo is not to be of giant proportions. I believe that such size regulation occurs during the early postimplantation period. It will be instructive to investigate how this regulation occurs and whether the geometrical consequences of such regulation may be part of the reason that these embryos die.

## **CHAPTER 7**

# **THE CONSEQUENCES OF TETRAPLOIDY IN THE TISSUES OF DEVELOPING MOUSE EMBRYOS**

## 7.1 INTRODUCTION

In earlier work, I showed that the volume of individual nucleated red blood cells of tetraploid mouse embryos was twice that of controls (Henery and Kaufman, 1992c). Such an increase in cell size, associated with a body size of similar proportions or indeed smaller than comparable diploid embryos, implied to me that the tissues of tetraploid embryos must contain cells which are more densely packed or they must contain less than the total number of cells found in diploid embryos. As a consequence of the latter hypothesis, the implications are that the tetraploid embryo does not alter the rate of growth of its constituent cells in the postimplantation period, since the implanting tetraploid blastocyst already contains half the cell number of diploid controls (Henery and Kaufman, 1991). This is in spite of the fact that it is well known that embryos, with a reduced number of cells, size regulate in the early postimplantation period so that their cell number is increased and they are eventually indistinguishable from controls (e.g. Tarkowski, 1959; Rands, 1986b). Here, I report on observations on the cell densities, cell sizes, and number of cells in various tissues in normal mouse embryos and in tetraploid embryos produced by electrofusion and discuss the effect that they may have on their normal development.

## 7.2 MATERIALS AND METHODS

### TETRAPLOID SERIES

Superovulated F1 hybrid female mice were mated with Rb(1.3)1Bnr male mice, and the embryos were flushed from the females at the two-cell stage. These embryos were subjected to an electrofusion stimulus to fuse the two blastomeres of the embryos together to produce one-cell tetraploid embryos, as described previously. These tetraploid embryos were unilaterally transferred to pseudopregnant recipients. The recipient mice were autopsied between day 12 and day 16 of gestation and the tetraploid embryos removed. The most advanced tetraploids were found to have a developmental age of between 13.5 and 14.5 days p.c.. For the work reported here, 11.5-14.5 day embryos were analysed. Cytogenetic analysis of the extra-embryonic membranes of these embryos unequivocally confirmed that these embryos had a homozygous tetraploid chromosome constitution (Kaufman and Webb, 1990).

### CONTROL SERIES

Naturally ovulating F1 hybrid females were mated with F1 hybrid males. The pregnant females were then autopsied between day 12 and day 15 of gestation to provide developmentally matched controls for the tetraploid series.

### EMBRYO SIZE

Only those tetraploid embryos that possessed a normal, postcranial vertebral axis were analysed in this study; this comprises about half of those isolated on days 15-16 of gestation (Kaufman, 1991a, 1992a). The developmental age of the tetraploid embryos was established by comparing their postcranial morphological/histological features with those of diploid controls. In specimens matched for developmental age, the unfixed crown-rump lengths of the tetraploid and control embryos were measured.

## MORPHOMETRIC ANALYSIS:

### ESTIMATING NUCLEAR VOLUME

The tetraploid and diploid embryos were processed for histology, as described previously. The volume of the nuclei of the cells were measured in the primitive ventral horn of developing neural tube between days 11.5-14.5 p.c., and the heart muscle cells, the cells of the mandibular mesenchyme tissue and the cells of the precartilage of the ear were analysed on day 14.5 p.c. material, as described previously.

### ESTIMATING CELLULAR DENSITY

In order to estimate the number of nuclei per unit volume from the numbers per unit area in the tissues analysed, Aherne's simplified formula (Aherne and Dunnill, 1982) was used as the diameter of the nuclei was of the same order of magnitude as the section thickness. This formula counts the number of cells per unit volume ( $N_v$ ):

$$N_v = \frac{2N_A}{i.d + 2t}$$

where  $N_A$  is the number of cells per unit area and  $t$  is the thickness of the sections. To calculate the value of  $i$ , parallel lines with a distance separation ( $d$ ) equivalent to 20 $\mu$ m were drawn on a transparency that was taped to the TV monitor;  $i$ , the frequency with which nuclei intersect these lines was then measured in selected fields. 20 $\mu$ m parallel lines were chosen because they were thought to be the most efficient size for intersecting a sufficient amount of cells for  $i$  to be calculated.



## 7.3 RESULTS

### 7.3.1 TETRAPLOID MORPHOLOGY AND SIZE

Tetraploid embryos are in many ways normal in appearance at 11 days p.c. The only striking difference between them and controls is that the two telencephalic lobes fail to separate normally, and all stages between complete holoprosencephaly (in which only a single cerebral hemisphere is formed) through partial separation of the telencephalic hemispheres are encountered (Kaufman and Webb, 1990). Later, there are clear craniofacial abnormalities involving the forebrain, eyes, and pituitary as well as characteristic facial appearance (Figure 7.1). Lesser abnormalities are found in the aortic arch arteries (Kaufman, 1992a) and about half of the developmentally most advanced embryos have an abnormal postcranial vertebral axis (Figures 7.1 g,h,i). In this group, the vertebral abnormalities are invariably associated with the presence of an enormous omphalocele (a large defect in the anterior abdominal wall covered only by a thin membrane composed of amnion and peritoneum) which contains the abdominal viscera, and occasionally the heart. At the histological level, however, the tissues of diploids and tetraploids are very similar and tetraploids differentiate as expected. Thus, for example, kidneys develop properly, showing well-developed glomeruli, gonads produce germ cells (Kaufman, 1991a), and limb development is normal. Tetraploid embryos are, however, some 10% shorter than normal embryos of equivalent developmental age (Table 7.1; Kaufman and Webb, 1990; Kaufman, 1991a).

### 7.3.2 MORPHOMETRIC MEASUREMENTS

As it was impractical to measure nuclear size and density in all tetraploid tissues, the tissues selected for analysis were chosen because they could easily be identified in sections, and because they represented a good range of developmental fates. They included cells of the ventral horn of the developing neural tube (Figures 7.2a-h; 7.3a, b), the developing heart muscle cells (Figures 7.3c and d), two types of mesenchyme, mandibular connective tissue (Figures 7.3e and f), and the precartilagae of the ear (Figures 7.3g and h). Nuclear size and density within the neural tube was measured in 11.5- to 14.5-day-old tetraploid embryos, and

**Figure 7.1**

Frontal and lateral views of three tetraploid embryos, two of which have a normal postcranial vertebral axial morphology (a-c, developmental age 13.5 days p.c.; d-f, 14.5 days p.c.), while a third embryo has an abnormal postcranial vertebral axis associated with an enormous omphalocele (g-i, approximately 14 days p.c.). Representative transverse sections through the cephalic region clearly display the grossly abnormal forebrain in these embryos, showing incomplete separation of the two telencephalic hemispheres (arrow; a, b X 6.5; d, e X 5; g, h X 8). (a, b: scale bar = 1.6mm; d, e, g, h: scale bar = 1.9mm; c, f, i: scale bar = 1mm)

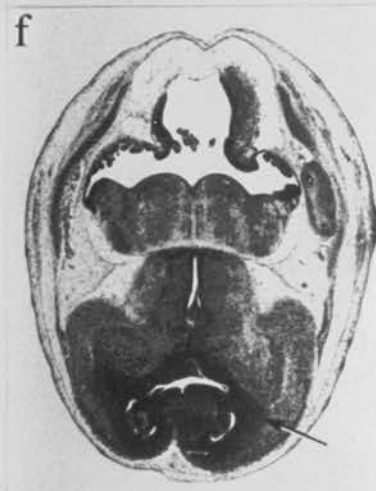
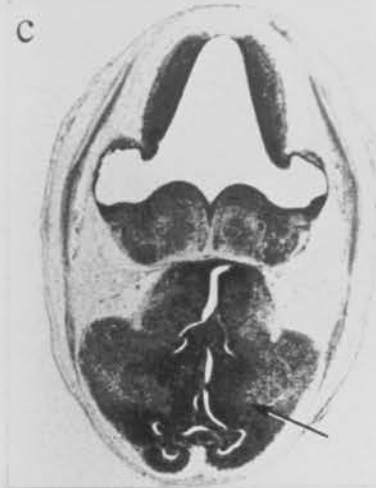


Table 7.1 Mean crown-rump lengths of tetraploid and diploid mouse embryos measured between 11.5-14.5 days p.c.

| Day of gestation<br>(d.p.c.) | No.tetraploid<br>embryos analysed | Tetraploid mean<br>unfixed crown-rump<br>length (mm)(± SEM) | No.diploid<br>embryos analysed | Diploid mean<br>unfixed crown-rump<br>length (mm)(± SEM) | 4N/2N<br>crown-rump length<br>(%) |
|------------------------------|-----------------------------------|---|--------------------------------|--|-----------------------------------|
| 11.5                         | 1                                 | 5.8*  | 10                             | 6.87 (± 0.06)  | 84.4%                             |
| 12.5                         | 3                                 | 7.4 (± 0.49)  | 9                              | 8.69 (± 0.14)  | 85.2%                             |
| 13.5                         | 2                                 | 9.4 (± 0.10)  | 9                              | 10.17 (± 0.14)   | 92.4%                             |
| 14.5                         | 2                                 | 11.45 (± 0.05)  | 9                              | 11.94 (± 0.17)   | 95.9%                             |

Table 7.2 Cross-sectional area of neural tube measured in the mid-thoracic region in tetraploid and developmentally matched diploid mouse embryos.

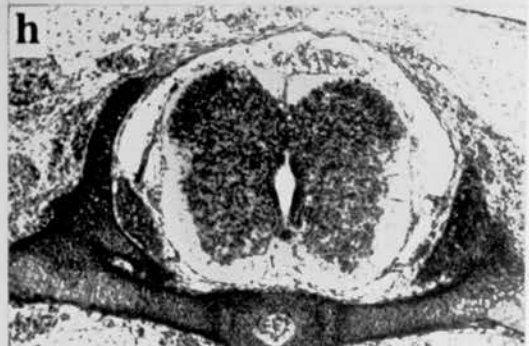
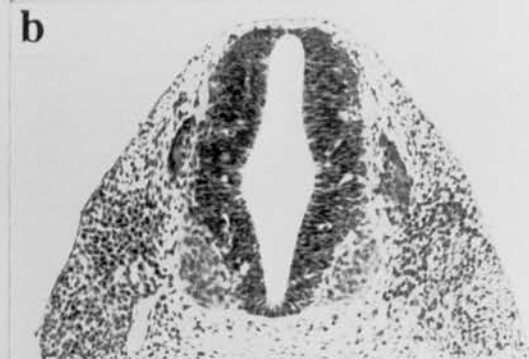
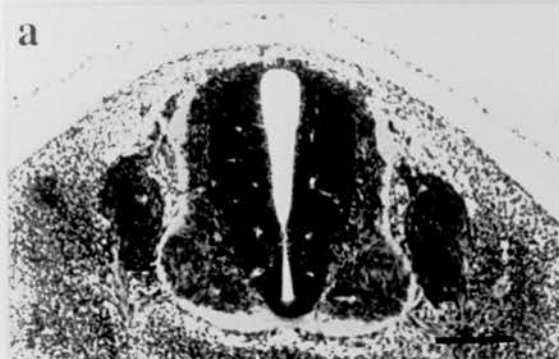
| Day of<br>gestation<br>(d.p.c.) | TETRAPLOID   |                           | DIPLOID  |                           | 4N:2N<br>ratio |
|---------------------------------|--|---------------------------|--|---------------------------|----------------|
|                                 | Mean neural tube area<br>( $\mu\text{m}^2$ ) (± SEM) | Total embryos<br>analysed | Mean neural tube area<br>( $\mu\text{m}^2$ ) (± SEM) | Total embryos<br>analysed |                |
| 11.5                            | 65872.5*   | 1                         | 63176.3 (± 4230.7)                                   | 2                         | 1.04:1         |
| 12.5                            | 99660.3 (± 11488.9)                                  | 3                         | 119347.3 (± 4907.1)                                  | 3                         | 0.83:1         |
| 13.5                            | 133027.7 (± 12746.7)                                 | 2                         | 130719.8 (± 8832.6)                                  | 3                         | 1.02:1         |
| 14.5                            | 147222.9 (± 8315.2)                                  | 2                         | 138066.5 (± 5659.8)                                  | 3                         | 1.07:1         |

\* No SEM as only one embryo measured

**Figure 7.2**

(a-h) Representative transverse sections through the neural tube (i.e., the developing spinal cord) in the mid-thoracic region in tetraploid embryos (b, d, f, h) and developmentally matched controls (a, c, e, g). a and b, 11.5 days p.c.; c and d, 12.5 days p.c.; e and f, 13.5 days p.c.; g and h, 14.5 days p.c.. The micrographs demonstrate that there is little if any difference in either the cross-sectional areas of tetraploid and diploid neural tubes or their degree of differentiation (a and b, X 100; c-h, X 63).

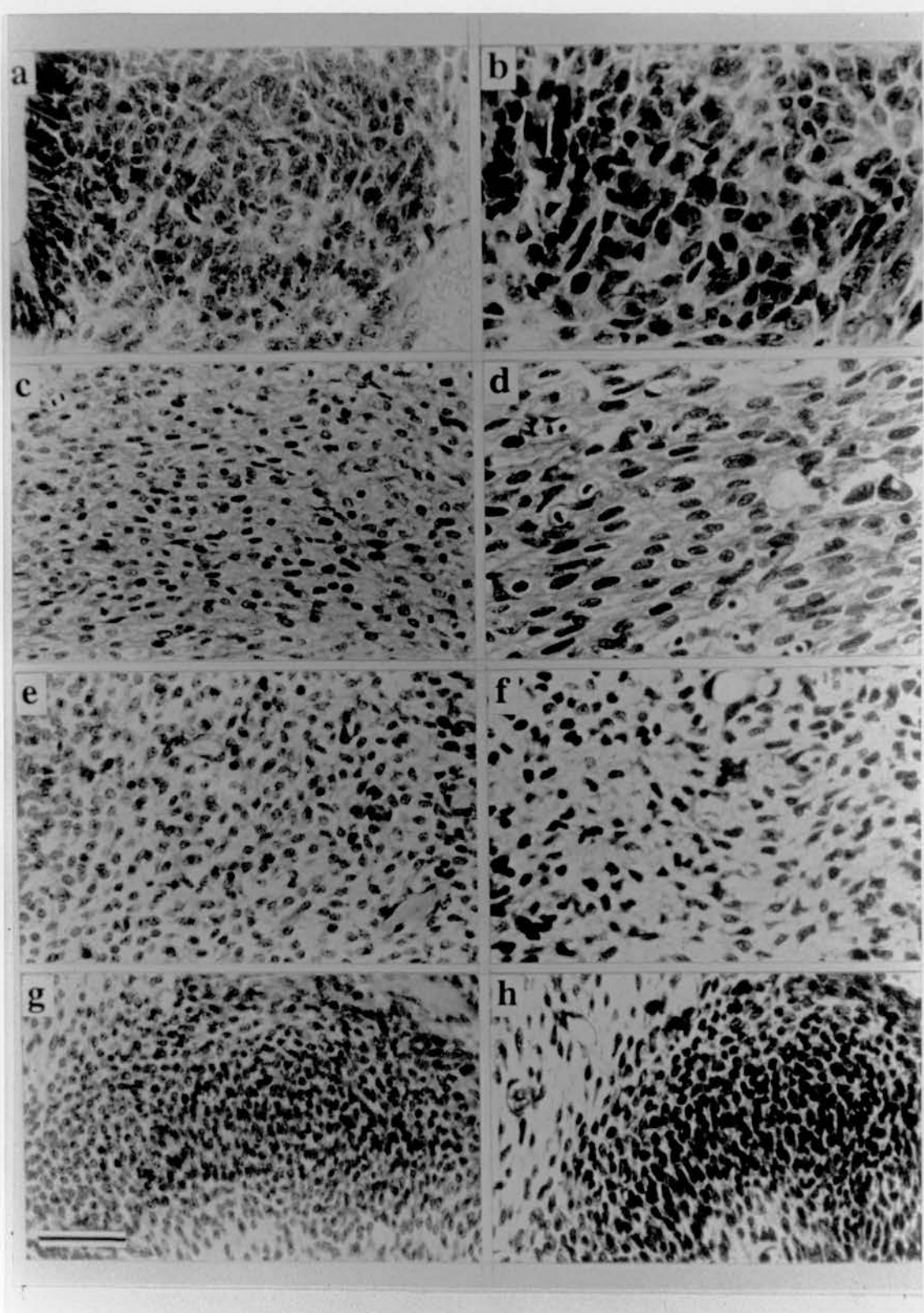
(a, b: scale bar = 150 $\mu$ m; c, d: scale bar = 200 $\mu$ m; e, f, g, h: scale bar = 300 $\mu$ m)



**Figure 7.3**

(a-h) Representative histological sections through a selection of tissues in a 14.5 days p.c. tetraploid embryo (b, d, f, h) and in a developmentally matched control embryo (a, c, e, g). a and b, cells of the ventral horn of the neural tube; c and d, heart muscle cells; e and f, mandibular mesenchyme tissue; g and h, precartilage of the ear. In each of the pairs, both nuclei (and cells) are clearly larger in tetraploids (right column) than in their diploid controls (left column). (All photomicrographs X 400; bar = 50 $\mu$ m).





the other tissues in 14.5-day-old tetraploids, and these were compared with equivalent measurements in controls. As the neural tube is so sharply delineated in transverse sections, its cross-sectional area was also measured at matched levels along the neural axis in both the tetraploids and the controls (Table 7.2).

The values for the volumes of tetraploid and control diploid cell nuclei are given in Table 7.3. In the cells of the ventral horn of the neural tube, the ratios of the nuclear volume over the period 11.5-14.5 days p.c. remains the same at about 2.34:1. The ratio for the heart muscle nuclei was a little higher at 2.53:1, while the values for the two connective tissues is a little smaller at 2.0:1. The estimate of the mean for all the tissues studied is  $2.28 \pm 0.16$ , or a little more than 2.

The numbers of intersections,  $i$ , (Table 7.4), and the number of nuclei per field were also counted. Since the nuclear diameters of the cells measured were of the same order of magnitude as the thickness of the tissue sections, it was possible to use Aherne's formula (see section 7.2) to estimate the density of cells per unit volume (Table 7.5). These figures are given in Table 7.5 and show clearly that the density of cells per unit volume in diploid embryos is a little over twice that in tetraploids, with the figures for the ventral horn cells of the neural tube being about 2.20:1, for the heart muscle cells 2.86:1, and the connective tissue mesenchyme about 2:1.

To estimate the ratio of the total number of cells in tetraploid and diploid embryos, we require to scale the ratios that are based on tissue sections to the volumes of the whole tissues and embryos. The best figures for calculations here are for the neural tube because the cross-sectional areas are about the same in the two types of embryos, but where the length ratio of tetraploids to diploids is about 0.9:1. Combining size and cell density ratios gives an estimate of the diploid-to-tetraploid ratio for the total number of cells in the neural tube of about 2.5:1. In short, it seems that the number of cells in tetraploid embryos is only about 40% of that in diploids.

Table 7.3 Nuclear volumes of the cells of tissues in tetraploid and diploid mouse embryos.

| Tissue                        | TETRAPLOIDS            |                      |   | DIPLOIDS               |                      |   |
|-------------------------------|------------------------|----------------------|---|------------------------|----------------------|---|
|                               | Total embryos analysed | Total cells analysed | Nuclear volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) | Total embryos analysed | Total cells analysed | Nuclear volume ( $\mu\text{m}^3 \pm \text{SEM}$ ) |
| Neural tube                   |                        |                      |   |                        |                      |   |
| 11.5 d.p.c.                   | 1                      | 77                   | 601.3*  | 2                      | 208                  | 241.8 ( $\pm 23.0$ )                              |
| 12.5 d.p.c.                   | 3                      | 291                  | 491.2 ( $\pm 105.6$ )                             | 3                      | 327                  | 216.3 ( $\pm 11.7$ )                              |
| 13.5 d.p.c.                   | 2                      | 177                  | 459.1 ( $\pm 55.4$ )                              | 3                      | 328                  | 222.2 ( $\pm 20.3$ )                              |
| 14.5 d.p.c.                   | 2                      | 169                  | 488.6 ( $\pm 54.1$ )                              | 3                      | 300                  | 192.2 ( $\pm 12.3$ )                              |
| Precartilage of ear           |                        |                      |   |                        |                      |   |
| 14.5 d.p.c.                   | 2                      | 215                  | 145.4 ( $\pm 11.4$ )                              | 3                      | 253                  | 78.2 ( $\pm 4.4$ )                                |
| Heart muscle                  |                        |                      |   |                        |                      |   |
| 14.5 d.p.c.                   | 2                      | 164                  | 300.4 ( $\pm 2.1$ )                               | 3                      | 274                  | 118.9 ( $\pm 20.0$ )                              |
| Manidibular connective tissue |                        |                      |   |                        |                      |   |
| 14.5 d.p.c.                   | 2                      | 188                  | 264.8 ( $\pm 45.2$ )                              | 3                      | 319                  | 126.5 ( $\pm 17.0$ )                              |
|                               |                        |                      |   |                        |                      | 4N:2N volume ratio                                |

\* No SEM as only one embryo analysed.

**Table 7.4** **Average values of i, intersections, for the cells of tissues**  
**in tetraploid and diploid embryos.**

|                     |          | <u>Tetraploid</u> | <u>Diploid</u> |
|---------------------|----------|-------------------|----------------|
| Neural tube         | day 11.5 | 0.68              | 0.73           |
|                     | day 12.5 | 0.74              | 0.66           |
|                     | day 13.5 | 0.79              | 0.58           |
|                     | day 14.5 | 0.75              | 0.56           |
| Heart muscle        | day 14.5 | 1.00              | 0.62           |
| Mandibular tissue   | day 14.5 | 0.73              | 0.47           |
| Precartilage of ear | day 14.5 | 0.51              | 0.38           |

The larger tetraploid values of i (with the exception of the neural tube at 11.5 days) reflect the larger size of their nuclei.

Table 7.5 Nuclear densities for the tissues of tetraploid and diploid mouse embryos.

| Tissue                       | TETRAPLOIDS   |   | DIPLOIDS  |   | $N_V$ 2N: $N_V$ 4N ratio |
|------------------------------|---|---|---|---|--------------------------|
|                              | No cells/area <sup>2</sup> ( $\mu\text{m}^2$ )<br>( $N_A$ ) | No cells/vol <sup>3</sup> ( $\mu\text{m}^3$ )<br>( $N_V$ )* | No cells/area <sup>2</sup> ( $\mu\text{m}^2$ )<br>( $N_A$ ) | No cells/vol <sup>3</sup> ( $\mu\text{m}^3$ )<br>( $N_V$ )* |                          |
| Neural tube                  |   |   |   |   |                          |
| 11.5 d.p.c.                  | 19.2  | 1.3   | 34.7  | 2.3   | 1.77:1                   |
| 12.5 d.p.c.                  | 18.4  | 1.2   | 42.0  | 2.9   | 2.42:1                   |
| 13.5 d.p.c.                  | 25.8  | 1.6   | 42.7  | 3.1   | 1.94:1                   |
| 14.5 d.p.c.                  | 18.9  | 1.2   | 43.8  | 3.2   | 2.67:1                   |
| Precartilage of the ear      |   |   |   |   |                          |
| 14.5 d.p.c                   | 53.7  | 4.1   | 84.3  | 7.1   | 1.73:1                   |
| Heart muscle                 |   |   |   |   |                          |
| 14.5 d.p.c.                  | 12.7  | 0.7   | 27.7  | 2.0   | 2.86:1                   |
| Mandibular connective tissue |   |   |   |   |                          |
| 14.5 d.p.c.                  | 19.5  | 1.3   | 35.4  | 2.8   | 2.15:1                   |

\* These figures are calculated using Abernethy's formula (see section 7.2)

## 7.4 DISCUSSION

The observations on tetraploid mouse embryos presented here clearly demonstrate that, in each of the tissues studied, there is a severely diminished number of cells that possess double-size nuclei; the embryos are, nevertheless, of almost normal size and their only consistently recognisable gross anatomical abnormality is a poorly developed forebrain (Kaufman and Webb, 1990; Kaufman, 1991a, b). Here, I briefly consider this data, the extent to which size regulation can take place in the developing mouse embryo, and the possible reasons that could lead to abnormal cerebral development as a primary defect with characteristic craniofacial abnormalities as their likely consequence. This in turn leads me to examine the relationship between global pattern-formation mechanisms and the cell milieu over which they operate.

### *Tetraploid survival*

Although plant, fish and amphibian tetraploids develop quite normally and can reproduce (Fankhauser, 1945; Myers et al., 1986), tetraploid mouse embryos do not survive well and only some 10% achieve the stage of hindlimb development (10.5 days p.c.). Of these, only the occasional embryo reaches day 16 of gestation, but are, in developmental terms, equivalent to normal embryos of about 14-14.5 days p.c. (Kaufman and Webb, 1990; Kaufman, 1991a, b, 1992a). In the case of humans, tetraploids also occasionally develop (Pajares et al., 1990) being usually represented as empty gestational sacs without any trace of embryonic rudiments (Carr, 1971b). Very rarely, tetraploid fetuses survive to term, and a total of seven liveborn have been reported that were full tetraploids, rather than diploid-tetraploid mosaics. Furthermore, tetraploid infants have been known to survive for up to two years (Lafer and Neu, 1988). The human tetraploids show a common and characteristic set of craniofacial abnormalities, but their postcranial features are not consistent between tetraploids.

Mouse tetraploid embryos, likewise, show few malformed organs and histological analysis reveals that those organs and tissues required for embryonic survival seem quite normal. Homozygous tetraploid rabbit embryos apparently also show few abnormalities, although Ozil and Modliński (1986) did find that the abnormal embryos showed abnormalities affecting the neural tube, particularly the brain region. For so few gross abnormalities, it is surprising that tetraploids die so early. The explanation may be quite straightforward, as tetraploid embryos develop poor placentas (M.H.Kaufman, personal communication) and thus have a limited oxygen supply. Normal placental circulation starts at around 9 days p.c. (Theiler, 1989), and the placenta itself becomes mature at about 12 days p.c. Inadequacies of perfusion in this tissue can thus impair growth and will inevitably lead to the death of the embryo, but are unlikely to have any other role (e.g. morphogenetic) in tetraploid development. This placental abnormality may be related to those that arise from imprinting when there are two maternally-derived sets of chromosomes (Barton et al., 1985; Moore and Haig, 1991). In humans, the placentas of heterozygous tetraploids (i.e. those with, for example, three sets of paternally-derived chromosomes and one maternally-derived set) can show partial hydatidiform degeneration (Sheppard et al., 1982; Surti et al., 1986), but this is not seen in the placentas of homozygous tetraploids. It would be instructive, therefore, to examine tetraploid embryos with diploid extra-embryonic tissues (produced for example, by injecting a tetraploid inner cell mass into a diploid trophoctoderm vesicle), to discover whether development of these chimeric embryos, with an improved placental tissue, is greatly enhanced.

In the light of these findings, it is particularly difficult to know how to interpret the significance of the claim that a number of cytochalasin B-induced, homozygous tetraploid mouse embryos were apparently capable of surviving to birth (Snow, 1973, 1975, 1976). Although the red blood cells of these embryos, for example, had a volume about four times larger than that of controls, no detailed morphometric comparisons were made with tissues from developmentally matched controls. Moreover, the great majority of these embryos were morphologically normal, if small as compared to controls, though haemorrhages were found in tissues of many organs. These, it was suggested, resulted from the mechanical stress involved



in circulating large nucleated red blood cells through vessels of normal diameter. While no consistent syndrome complex was reported, abnormalities involving the forebrain and its derivatives were commonly encountered (e.g. Figure 7.1). The difference between Snow's observation and the most recent experiments, could reflect strain variability, but a more likely explanation is that some or possibly all of his most advanced embryos were, in fact, diploid-tetraploid mosaics rather than pure tetraploids, despite the fact that cytogenetic analysis failed to demonstrate this in more than a few instances. It is perhaps unfortunate that information regarding placental morphology was not included in the original report (Snow, 1975), since this tissue was isolated, weighed and fixed for histological examination.

It should be noted that the method used by Snow to induce tetraploidy involved the prolonged exposure of 2-cell stage embryos to cytochalasin B in order to inhibit the second cleavage, the efficiency of the technique being about 40-75%. Of the assumed homozygous tetraploids produced by this means, about half developed to the blastocyst stage *in vitro* and these were then transferred to the uteri of recipients. This technique is inherently less reliable than the use of electrofusion to achieve tetraploidy: here, only 2-cell embryos that have reverted to a single cell and so have unequivocally diploidised to form homozygous, tetraploid embryos are transferred to recipients.

#### *Regulation successes and failures*

In common with those of other species (Beatty, 1957), mouse tetraploid tissues have large cells with nuclear volumes about twice the size of their diploid equivalents (Henery and Kaufman, 1992c). This is so for all tissues examined and is irrespective of their absolute cell types and densities (Tables 7.3 and 7.5). The cell volume in tetraploids is a little more than twice that in diploids, irrespective of whether the cells are small (e.g. precartilage of the ear and mandibular mesenchyme), or large (e.g. ventral horn cells), and there are less than half the number of cells in tetraploids as compared to controls. The mechanism by which chromosome complement controls nuclear and cell size in postimplantation embryos remains unknown. A consequence of such an increase in cell and nuclear volume should be an increase in body size, but like the

amphibian polyploids (see, for example Sperry, 1988), body size is often the same or smaller than diploids. It is possible that polyploid embryos size-regulate so that large cells divide less frequently than normal sized cells. This may occur in triploid embryos which have the same number of cells at implantation as diploids, but might not occur in tetraploids formed from the inhibition of a cleavage division (or, as here, formed by fusion of two diploid blastomeres to one tetraploid blastomere), since these embryos already have half the cell number of diploid embryos. It is well known that large diploid embryos, formed from aggregates of cells, also size regulate by reducing their total cell number so that a normal sized embryo is formed, and that this regulation occurs early in the postimplantation period (Tarkowski, 1963; Mintz, 1971; Buehr and McLaren, 1974; Lewis and Rossant, 1982; Rands, 1986a). The mechanism of such regulation in these embryos is unclear, but it may result from a change in the growth rate of the cells of the embryo. Most growth in dry weight in the mouse embryo occurs at around day 6-10 (McLaren, 1976), a delay in this growth spurt would also reduce the total number of cells in these embryos (Rands, 1986a).

As tetraploid embryos have less than half the number of cells of diploids, it is surprising that the histological appearance of tetraploid tissues is almost indistinguishable from that of controls. Normal germ cells, ovaries and testes form (Kaufman, 1991a), the fine detail of kidney structure is as expected, and one has to look for such fine detail as the exact pattern of the branchial/pharyngeal arch arteries before it is possible to discern that tissue development can go awry in some tetraploids (Kaufman, 1992a). Indeed, although there are fewer cells in these embryos, the coarseness of cellular organisation is, in general, only apparent in cell counts, and not in morphology.

The normality of tetraploid tissue organisation is not matched by the gross appearance of the embryos. Here, there is one consistent difference between tetraploids and controls and that is the development of the forebrain. In all cases, this reflects incomplete morphogenesis of the two cerebral hemispheres even though the mid- and hindbrain regions, in most instances, seem

relatively normal. On the basis of their external appearance, tetraploid humans likewise show abnormal forebrain development: they tend to have microcephaly, narrow foreheads, microphthalmia and high-arched and/or cleft palates (Pajares et al., 1990).

That mammalian tetraploids should have abnormal forebrains, and not share any other consistently identifying feature is surprising. The reasons for the abnormality clearly lie neither in an inability to grow (the remaining brain, spinal cord and all other non-neural tissues achieve a normal size), nor in problems associated with differentiation *per se* (neurons develop normally), nor in inadequate morphogenetic mechanisms (there are no such mechanisms that are unique to the forebrain and not employed elsewhere (Bard, 1990)). I, therefore, seem driven to the conclusion that the reasons for this feature derive from the fact that the pattern-formation mechanisms underpinning tetraploid forebrain development go awry in this tissue alone. Moreover, this fault may be limited to the Mammalia, as tetraploid trout and amphibia possess brains that seem to be morphologically and functionally indistinguishable from those of diploids (Myers et al., 1986).

The reasons for the abnormal forebrain development are unknown. It could lie in the fact that such dramatic changes in cellular size and spatial relationships present the embryo with too many geometrical problems (Epstein, 1986), but this should occur in other tissues too. There is one obvious possibility that merits consideration given that tetraploids have only half the requisite number of cells in this tissue. The abnormality could arise because there are just too few cells in the forebrain for the necessary functions to be separately distributed to the tissue by the underlying pattern-formation mechanism responsible for brain development. The cellular grain could be just too coarse, and the number of cells too few for normal development to take place, and the net result is that individual cells are assigned excessive or contradictory tasks and so are unable to participate properly in contributing to the developing brain. Evidence compatible with this view can be found in the early experiments of Snow and Tam (1979) using mitomycin C (MMC). MMC reduced the number of cells in treated embryos to about 14% of the normal at 7.5 days, and the single most common feature found in 26% of all

14-day-old newborn mice subjected to this treatment was microphthalmia. Furthermore, lower tetraploid vertebrates, whose forebrains are less sophisticated, develop normally. It is conceivable that such complexity of tasks is unique to this mammalian developing tissue and that it is most sensitive to such changes in cell size and number.

## **CHAPTER 8**

### **THE INCIDENCE OF ANEUPLOIDY AFTER SINGLE PULSE ELECTROACTIVATION OF MOUSE OOCYTES**

## 8.1 INTRODUCTION

The exposure of oocytes to pulses of electricity appears to be an effective activating stimulus in mice (Tarkowski et al., 1970; Witkowska, 1973 a,b; Gulyas, 1976; Onodera and Tsunoda, 1989; Didion et al., 1990; Landa and Hajkova, 1990; Marcus, 1990; Rickords and White, 1992; Vitullo and Ozil, 1992), rabbits (Gulyas, 1976; Ozil, 1990; Fissore and Robl, 1992), hamsters (Kaufman et al., 1975; Gulyas, 1976), pigs (Prather et al., 1989; Sun et al., 1992) and cattle (Ware et al., 1989). This stimulus is also widely used in nuclear transplantation studies to fuse either a donor blastomere or nucleoplast fragments to a recipient oocyte and at the same time activate the latter (Stice and Robl, 1988; Collas et al., 1989; Robl and Stice, 1989). Recently this experimental procedure has been used to simulate events at fertilization, in which intracellular  $\text{Ca}^{2+}$  oscillations occur, and the level of electrical stimulation employed has been found to have a significant effect on the development potential of the resultant parthenogenones (Ozil, 1990).

It is relevant to establish whether aneuploidy may occur as a direct or indirect consequence of electrical stimulation of oocytes. The brief exposure of oocytes to a dilute solution of ethanol, for example, is also a particularly effective activating stimulus (Kaufman, 1982; Cuthbertson, 1983; O'Neill and Kaufman, 1989), but a relatively small (10-20%) but nevertheless significant proportion of the resultant parthenogenones are found to be aneuploid (Kaufman, 1982; O'Neill and Kaufman, 1989; O'Neill et al., 1989). Strontium chloride is also an effective activating stimulus but, unlike ethanol, induces relatively low rates of aneuploidy (O'Neill et al., 1991).

Changes associated with postovulatory ageing *in vivo* are believed to also lead to an increase in the incidence of chromosomal segregation errors at the second meiotic division in fertilized mouse embryos (Eichenlaub-Ritter et al., 1986), but this does not appear to be an invariable finding in relation to either *in vivo* or *in vitro* aged mouse oocytes (Donahue and Karp, 1973; Badenas et al., 1989). No increase in the incidence of aneuploidy was observed when

postovulatory aged mouse oocytes were activated parthenogenetically with hyaluronidase (O'Neill and Kaufman, 1988). These observations would seem to indicate that neither parthenogenesis *per se*, nor postovulatory ageing of the oocyte, is invariably associated with aneuploidy. However, postovulatory ageing of oocytes prior to their fertilization, is associated with an increased incidence of polyploidy (e.g. Vickers, 1969; Badenas et al., 1989).

It is well documented that postovulatory aged oocytes activate more readily than recently ovulated oocytes (Kaufman, 1973a; Webb et al., 1986; O'Neill and Kaufman, 1988; Shaw and Trounson, 1989). The postovulatory age of the oocyte at the time of activation also influences the incidence of the various classes of parthenogenone induced (Kaufman, 1973a; Kaufman and Surani, 1974), and this is most likely to be a consequence of the age-related changes that occur in the location of the meiotic spindle apparatus, the cortical granules, and the state of polymerisation of the cytoskeletal elements (Szöllösi, 1971, 1975; Longo, 1980). The retention of both products of the second meiotic division may lead to the production of diploid parthenogenones, and a higher proportion of such embryos develop to the blastocyst stage and into the early postimplantation period than haploid parthenogenones (Henery and Kaufman, 1992a).

Since over the last few years electrical stimulation has become increasingly widely used for the production of parthenogenones, it is of interest to analyse in detail the immediate cytogenetic response of cumulus-denuded oocytes to a constant pulse of electrical stimulation. The use of a single pulse activation stimulus contrasts with a recent preliminary study of one- pronuclear haploid parthenogenones which resulted from repetitive pulse stimuli involving recently ovulated oocytes (Vitullo and Ozil, 1992). In that paper, a low level of aneuploidy was observed, but no mention was made whether this was due to hypo- or hyperhaploidy. I have therefore investigated whether electrical pulses *per se* have any effect on the incidence of aneuploidy at a variety of postovulatory ages.



## 8.2 MATERIALS AND METHODS

### THE ELECTROACTIVATION OF CUMULUS-FREE OOCYTES

8- to twelve-week-old F1 hybrid female mice were superovulated. At 15.5, 18.5, 22.5 and 25 hours after the HCG injection the mice were killed and their cumulus masses containing the ovulated oocytes were recovered. Their adhering cumulus cells were removed with hyaluronidase and these cumulus-denuded oocytes were then subjected to electroactivation, as described previously. The embryos were removed immediately and washed and cultured for 4-5 hours.

Thereafter, upon activation, four classes of parthenogenone could be determined (see section 2.21), but only the single-pronuclear haploid parthenogenones (1PN) were studied further. They were transferred to colcemid to arrest their development at metaphase of the first cleavage division, and early the next morning chromosome spreads were prepared by the air-drying technique described by Tarkowski (1966). After staining, the chromosome constitution of each preparation was determined.

### CONTROL GROUP: SHAM ELECTROACTIVATION OF CUMULUS-FREE OOCYTES

In this group, F1 hybrid mice were superovulated as described above, and at 15.5, 18.5, 22.5 and 25 hours after the hCG injection were killed. Cumulus-free oocytes were transferred into a non-electrolyte solution and then into the electroactivation chamber containing the same solution, but no pulse was triggered. After 5-6 hours in culture, a proportion of the oocytes had activated. Only the single-pronuclear parthenogenones were further cultured in colcemid, and early the next morning chromosome spreads were prepared and stained as described above.

#### CONTROL GROUP: HANDLING OF OOCYTES

F1 hybrid mice were superovulated and the cumulus masses recovered at the various times after the HCG injection as indicated above. Their adhering cumulus cells were removed with hyaluronidase, and after further culture for 5-6 hours, their rate of activation was recorded.

#### CONTROL GROUP: FERTILIZED OOCYTES

An additional group of F1 hybrid mice were superovulated and caged individually with fertile homozygous Rb(1.3)1Bnr male mice immediately after the HCG injection. Thus, following fertilization and incubation in colcemid, a first cleavage metaphase spread (technically this is a C-mitotic spread) containing two distinct groups of chromosomes allows the female and male chromosome sets to be distinguished. At 6-8 hours after the expected time of fertilization, the females were killed, and the fertilized embryos recovered. After 5-6 hours in culture, they were transferred into colcemid in order to arrest them at metaphase of the first cleavage division. Chromosome spreads were prepared and analysed as described earlier. In the absence of incubation in colcemid, a single first cleavage metaphase spread would be obtained because the two (i.e. male and female) pronuclear chromosomes would have amalgamated together on the equator of the first cleavage spindle. The latter is destroyed (by depolymerisation) by colcemid, so that the two chromosome sets retain their individuality.

A small proportion of embryos from all the groups were impossible to analyse due to overlapping of the chromosomes in the spread, indistinguishable male and female chromosome sets, scattering of chromosomes, and in a few cases interphase nuclei were recorded instead of metaphase spreads. In these cases the recording was non-analysable (NA).

To account for the fact that the loss of chromosomes due to technical reasons may increase the level of hypohaploid preparations, an adjusted rate of aneuploidy was used, as described earlier. The latter is, of course, based on the assumption that there would be expected to be an equal incidence of hypo- and hyperhaploid preparations due to the random segregation of individual chromosomes to one or other pole of the spindle.

## 8.3 RESULTS

### 8.3.1 INCIDENCE OF ANEUPLOIDY

#### EFFECT OF ELECTROACTIVATION ON THE CHROMOSOME CONSTITUTION OF SINGLE-PRONUCLEAR HAPLOID PARTHENOGENONES AT METAPHASE OF THE FIRST CLEAVAGE DIVISION

The effect that electroactivation has on the chromosome constitution of single- pronuclear embryos is shown in Table 8.1. At 15.5, 18.5, 22.5, and 25h after hCG , the adjusted frequency of aneuploidy is 1.2, 4.0, 1.0, and 2.4%, respectively. The results obtained from the chromosome analyses of embryos that were not exposed to the electric pulse are shown in Table 8.2. At comparable times to the former group, the adjusted frequencies were 0, 2.8, 3.2, and 3.2%, respectively. Thus, there was no significant difference in the incidence of aneuploidy between the experimental group of single-pronuclear haploid parthenogenones and the control group of parthenogenones ( $p > 0.05$ ). These results demonstrate that electroactivation *in vitro* does not increase the incidence of chromosomal segregation errors as evidenced at the first cleavage mitosis. Representative first cleavage metaphase preparations of euploid and aneuploid single-pronuclear parthenogenetic embryos are shown in Figure 8.1.

#### CHROMOSOME CONSTITUTION OF *IN VIVO* FERTILIZED EMBRYOS AT METAPHASE OF THE FIRST CLEAVAGE DIVISION

Out of 336 maternally-derived chromosome preparations that were analysed in this study, only 1 was hyperhaploid and this gave an adjusted aneuploidy rate of 0.61% (see Table 8.3). Most of the aneuploid preparations encountered were hypohaploid, but the majority of these can probably be accounted for by technical artefacts. 1.8% of the fertilized embryos were triploid (one diandric, three digynic and two triploids of unknown parental origin).

TABLE 8.1  
Effect of electroactivation on the chromosome constitution of single-pronuclear haploid parthenogenones at metaphase of the first cleavage division.

| Group | Postovulatory age (hours) | No.chromosome spreads | No.spreads analysable | chromosome constitution |    |     |       | Adjusted rate aneuploidy(%) |
|-------|---------------------------|-----------------------|-----------------------|-------------------------|----|-----|-------|-----------------------------|
|       |                           |                       |                       | 18                      | 19 | 20  | 21 22 |                             |
| 1     | 15.5                      | 192                   | 170                   | 3                       | 10 | 156 | 0 1   | 1.2                         |
| 2     | 18.5                      | 375                   | 297                   | 2                       | 32 | 257 | 5 1   | 4.0                         |
| 3     | 22.5                      | 243                   | 198                   | 3                       | 9  | 184 | 1 0   | 1.0                         |
| 4     | 25.0                      | 210                   | 165                   | 1                       | 12 | 149 | 2 0   | 2.4                         |

TABLE 8.2  
Chromosome constitution of single-pronuclear haploid parthenogenones at metaphase of the first cleavage division after control "sham" electroactivation.

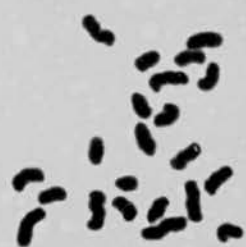
| Group | Postovulatory age (hours) | No.chromosome spreads | No.spreads analysable | chromosome constitution |    |     |       | Adjusted rate aneuploidy(%) |
|-------|---------------------------|-----------------------|-----------------------|-------------------------|----|-----|-------|-----------------------------|
|       |                           |                       |                       | 18                      | 19 | 20  | 21 22 |                             |
| 1     | 15.5                      | 36                    | 33                    | 0                       | 1  | 32  | 0 0   | 0.0                         |
| 2     | 18.5                      | 257                   | 217                   | 4                       | 13 | 197 | 2 1   | 2.8                         |
| 3     | 22.5                      | 291                   | 186                   | 1                       | 8  | 174 | 3 0   | 3.2                         |
| 4     | 25.0                      | 303                   | 190                   | 3                       | 13 | 171 | 2 1   | 3.2                         |

**Figure 8.1**

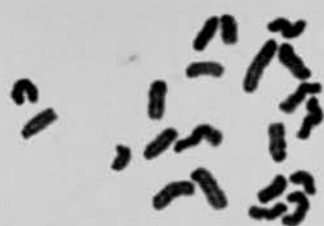
Representative first cleavage metaphase preparations of euploid and aneuploid single-pronuclear parthenogenetic embryos. (scale bar = 15 $\mu$ m)

- A) Aneuploid parthenogenetic embryo with 22 chromosomes.
- B) Aneuploid parthenogenetic embryo with 21 chromosomes.
- C) Euploid parthenogenetic embryo with 20 chromosomes.
- D) Aneuploid parthenogenetic embryo with 19 chromosomes.

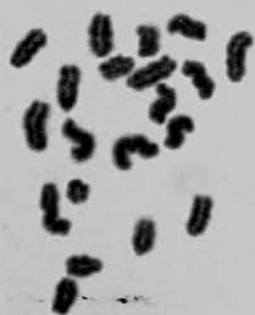
A



B



C



D

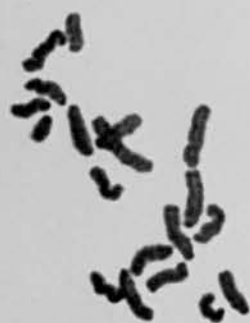


TABLE 8.3

Chromosome constitution of *in vivo* fertilized embryos (F1 hybrid x Rb(1.3)1Bnr) at metaphase of the first cleavage division.

| Group                         | No. analysable preparations * | chromosome constitution |     |     |    | Adjusted rate aneuploidy(%) |
|-------------------------------|-------------------------------|-------------------------|-----|-----|----|-----------------------------|
|                               |                               | 18                      | 19  | 20  | 21 | 22                          |
| female (F1 hybrid) pronucleus | 330                           | 3                       | 11  | 315 | 1  | 0                           |
| male (Rb(1.3)1Bnr) pronucleus | 326                           | 17                      | 303 | 6   | 0  | 0                           |
|                               |                               |                         |     |     |    | 3.7                         |

\* The total number of fertilized 1-cell stage embryos examined was 582, of which 6 were triploid.

TABLE 8.4

The effect of electroactivation on parthenogenetic activation rates and developmental pathways in relation to postovulatory age.

| Group | Postovulatory age (hours) | No.oocytes recovered | No.oocytes activated | Activation rate (%) | Developmental pathways* |     |    |      |
|-------|---------------------------|----------------------|----------------------|---------------------|-------------------------|-----|----|------|
|       |                           |                      |                      |                     | IPN                     | 2PN | IC | IPND |
| 1     | 15.5                      | 406                  | 234                  | 57.6                | 192                     | 15  | 19 | 8    |
| 2     | 18.5                      | 439                  | 398                  | 90.7                | 375                     | 11  | 9  | 3    |
| 3     | 22.5                      | 316                  | 304                  | 96.2                | 243                     | 41  | 15 | 5    |
| 4     | 25.0                      | 644                  | 543                  | 84.3                | 382                     | 76  | 84 | 1    |

\* 1PN = single-pronuclear haploid parthenogenone  
 2PN = 2 pronuclear (diploid) parthenogenone  
 IC = immediate cleavage parthenogenone  
 IPND = single-pronuclear diploid parthenogenone  
 (for further details see text)



### 8.3.2 ACTIVATION RATES

#### THE EFFECT OF ELECTROACTIVATION ON THE RATE OF PARTHENOGENETIC ACTIVATION

The activation rate of oocytes recovered at 15.5, 18.5, 22.5 and 25 hours after the hCG injection and subjected to an electrical pulse is shown in Table 8.4. The activation rates of the oocytes not exposed to an electrical pulse in the sham electroactivation groups are shown in Table 8.5. In the 15.5 hours group, the level of activation is approximately eight times higher in oocytes exposed to an electrical pulse than in those which were not (57.6% v 7.3%), and this difference is highly significant ( $p < 0.001$ ). At all other time points, the activation rate of oocytes in the experimental group is significantly higher than the control group ( $p < 0.001$ ).

#### THE EFFECT OF HANDLING AND EXPOSURE TO HYALURONIDASE ON THE RATE OF PARTHENOGENETIC ACTIVATION

The activation rate of oocytes in response to handling and exposure to hyaluronidase increased over time, and these findings are shown in Table 8.6. The activation rate of oocytes recovered at hCG + 15.5, 18.5, 22.5, and 25 hours was 0, 9.4, 29.8, and 68.4% respectively.

### 8.3.3 THE EFFECT OF ELECTROACTIVATION ON PARTHENOGENETIC DEVELOPMENTAL PATHWAYS

The incidence of the various pathways of parthenogenetic development that were observed after isolation of the oocytes at hCG + 15.5, 18.5, 22.5 and 25 hours in both the experimental and control groups is shown in Figure 8.2. The highest percentage of activated oocytes in both groups belonged to the single-pronuclear haploid parthenogenetic class (1PN). A comparison of the pathways observed at the various time points studied in oocytes activated by an electrical pulse and those which were not, revealed that there was a significant difference between them ( $p < 0.05$ ). Representatives of all of the four classes of parthenogenones were always found in the experimental groups, but this finding was only observed in the controls in the hCG + 25

**TABLE 8.5**  
Parthenogenetic activation rates and developmental pathways after "sham" electroactivation in relation to postovulatory age.

| <u>Group</u> | <u>Postovulatory age (hours)</u> | <u>No.oocytes recovered</u> | <u>No.oocytes activated</u> | <u>Activation rate (%)</u> | <u>Developmental pathways*</u> |            |           |             |
|--------------|----------------------------------|-----------------------------|-----------------------------|----------------------------|--------------------------------|------------|-----------|-------------|
|              |                                  |                             |                             |                            | <u>1PN</u>                     | <u>2PN</u> | <u>IC</u> | <u>1PND</u> |
| 1            | 15.5                             | 988                         | 72                          | 7.3                        | 67                             | 5          | 0         | 0           |
| 2            | 18.5                             | 729                         | 273                         | 37.4                       | 257                            | 15         | 0         | 1           |
| 3            | 22.5                             | 462                         | 331                         | 71.6                       | 291                            | 38         | 0         | 2           |
| 4            | 25.0                             | 709                         | 488                         | 68.8                       | 410                            | 52         | 23        | 3           |

\* see Table 8.4.

**TABLE 8.6**  
Parthenogenetic activation rates and developmental pathways after handling and exposure to hyaluronidase.

| <u>Group</u> | <u>Postovulatory age (hours)</u> | <u>No.oocytes recovered</u> | <u>No.oocytes activated</u> | <u>Activation rate (%)</u> | <u>Developmental pathways*</u> |            |           |             |
|--------------|----------------------------------|-----------------------------|-----------------------------|----------------------------|--------------------------------|------------|-----------|-------------|
|              |                                  |                             |                             |                            | <u>1PN</u>                     | <u>2PN</u> | <u>IC</u> | <u>1PND</u> |
| 1            | 15.5                             | 101                         | 0                           | 0                          | 0                              | 0          | 0         | 0           |
| 2            | 18.5                             | 139                         | 13                          | 9.4                        | 12                             | 1          | 0         | 0           |
| 3            | 22.5                             | 57                          | 17                          | 29.8                       | 16                             | 1          | 0         | 0           |
| 4            | 25.0                             | 95                          | 65                          | 68.4                       | 49                             | 14         | 2         | 0           |

\* see Table 8.4.

Figure 8.2

Histograms which demonstrate the proportionate incidence of the various developmental pathways of parthenogenetic embryos observed in relation to postovulatory age after electroactivation (experimental) and "sham" electroactivation (control). Figures a, b, c, and d refer to hCG + 15.5, + 18.5, + 22.5 and + 25 hours, respectively.

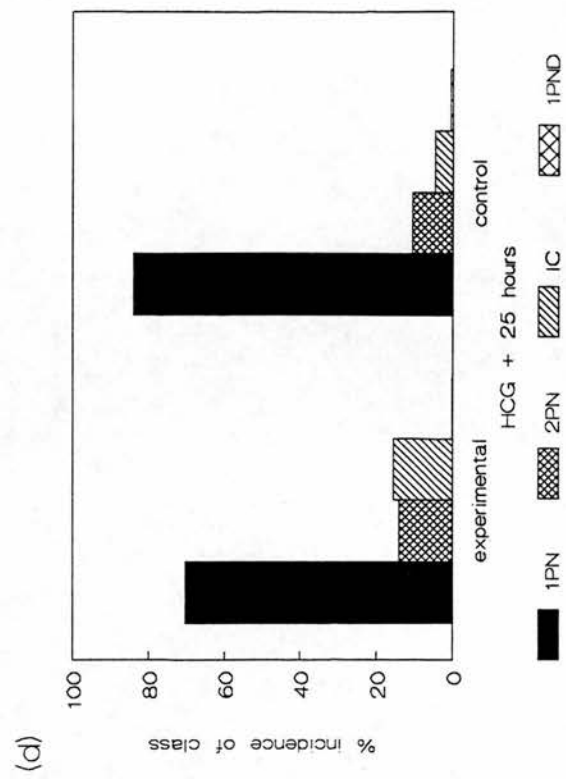
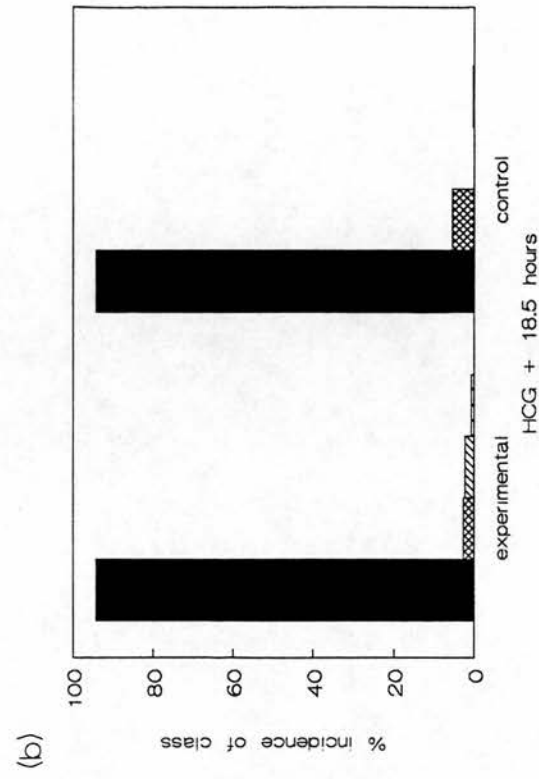
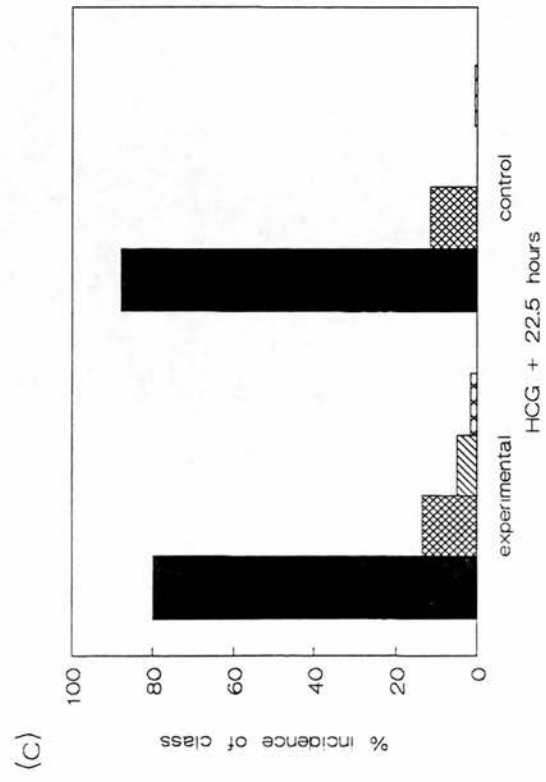
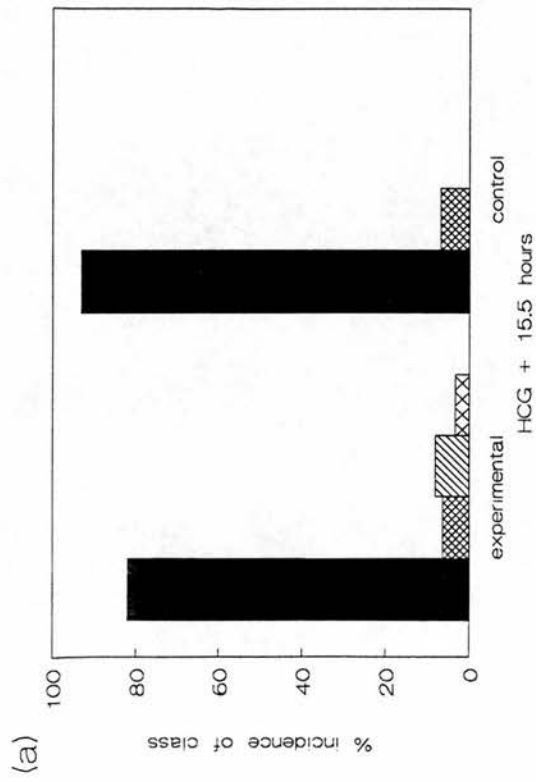
1PN = single-pronuclear (haploid) parthenogenone

2PN = two-pronuclear (diploid) parthenogenone

IC = "immediate cleavage" parthenogenone

1PND = single-pronuclear diploid parthenogenone

Figure 8.2



hours group, with the occurrence of some IC embryos. At this time point, only 70% of the parthenogenones are of the 1PN class in the experimental group, whereas 84% of the control group are of this type. It appears therefore, that the electrical stimulus induces oocytes to progress along different developmental pathways to those normally observed in the control group.

#### **8.3.4 EFFECT OF POSTOVULATORY AGEING ON PARTHENOGENETIC DEVELOPMENT**

The activation rate of oocytes that were isolated at hCG + 15.5 hours was significantly lower ( $p < 0.001$ ) than in oocytes isolated at hCG + 18.5, 22.5 and 25 hours in both the experimental and control groups (Tables 8.4 and 8.5). In addition, the activation rate of oocytes isolated at hCG + 18.5 hours was significantly different from oocytes isolated at 22.5 hours in both groups ( $p < 0.01$ ). The activation rate increased with the postovulatory age of the oocyte, and peak activation rates were achieved in both groups at hCG + 22.5 hours, after which time the activation rates decreased. No significant difference was observed in the incidence of aneuploidy in relation to increasing postovulatory age of the oocyte in either the experimental or the control groups (Tables 8.1 and 8.2).

## 8.4 DISCUSSION

Unfertilized oocytes can undergo parthenogenetic development following activation by a wide range of physical or chemical stimuli (Kaufman, 1983). Electroactivation is a successful stimulus, and is widely used in experiments designed to clone animals. The electrical pulse used to fuse a donor nucleus to a recipient oocyte will also often simultaneously activate that oocyte and enable reprogramming of the donor nucleus as well as stimulating its subsequent development (Stice and Robl, 1988; Robl and Stice, 1989). It is also a useful stimulus in that it simulates events that normally occur at fertilization. In the mouse and hamster, the activation stimulus provided by the fertilizing sperm produces (among other events) an elevation in intracellular  $\text{Ca}^{2+}$  levels followed by further smaller pulses of  $\text{Ca}^{2+}$  (Cuthbertson et al., 1981; Cuthbertson and Cobbold, 1985; Miyazaki, 1988). It is also clear that the resumption of meiosis and cortical granule exocytosis is dependent on this intracellular rise in  $\text{Ca}^{2+}$  (Kline and Kline, 1992). Most parthenogenetic stimuli only produce a single large but transient increase in intracellular  $\text{Ca}^{2+}$ . It was recently reported that repetitive pulse stimulation (as opposed to the production of a single pulse) in medium containing  $\text{Ca}^{2+}$ , can very closely simulate the intracellular ionic oscillations that normally occur at fertilization (Ozil, 1990; Vitullo and Ozil, 1992). However, Sun et al. (1992) have described the electroactivation of porcine oocytes, and they suggest that while multiple stimulations can result in cortical granule exocytosis and protein reprogramming in the activated parthenogenetic embryos, these stimulations differ markedly from those induced by spermatozoa at fertilization.

Some effective parthenogenetic agents such as ethanol can, however, induce the production of high rates of aneuploidy in the resultant embryos (Kaufman, 1982; O'Neill and Kaufman, 1989), and I was interested to establish whether a single electrical activation pulse had a similar effect. My findings establish that no significant difference in the incidence of aneuploidy is observed between oocytes that were electrically activated and those in the control groups at any of the postovulatory ages analysed. Clearly, the present electroactivation and postovulatory ageing study (see also O'Neill and Kaufman, 1988) indicates that these factors

have little effect on the induction of aneuploidy. It has been hypothesised that postovulatory ageing of oocytes in humans may be the cause of the increased incidence of meiotic errors observed in fertilized embryos (German, 1968), but this hypothesis has not been subjected to critical analysis. In mice, postovulatory ageing of oocytes does not appear to increase the incidence of meiotic errors (Donahue and Karp, 1973; Badenas et al., 1989). Vitullo and Ozil (1992), in a much smaller study of electrically activated single-pronuclear haploid parthenogenones than that presented here, noted that at hCG + 12 hours, 4 of 59 activated oocytes (ie 7%) displayed evidence of aneuploidy, though no indication was given whether this was due to hypo- or hyperhaploidy. Since it is now well established that poor spreading of preparations can lead to loss of chromosomes, it is difficult to know how to interpret these findings.

Parthenogenesis *per se* does not increase the susceptibility of oocytes to undergo malsegregation events, as low rates of aneuploidy are observed following hyaluronidase-induced (O'Neill and Kaufman, 1988) and strontium-induced activation (O'Neill et al, 1991). When recently ovulated oocytes are exposed to ethanol, however, high rates of aneuploidy are induced, and the rates of aneuploidy observed appear to be directly related to the duration of exposure to this agent (Kaufman, 1982; O'Neill and Kaufman, 1988). Furthermore, ultrastructural analyses of oocytes which have been exposed to ethanol have revealed the presence of lagging chromosomes and anomalies of the meiotic spindle apparatus in a proportion of them (O'Neill et al., 1989).

One of the first responses to a single electrical activation pulse in the mouse is the rotation of the spindle apparatus to a radial position (Gulyas, 1976). While the latter study demonstrated ultrastructural damage to the spindle apparatus and abnormal separation of the chromosomes at anaphase, no follow-up study was unfortunately undertaken to establish the cytogenetic consequences of these observations. It is therefore unclear at the present time, whether transient or irreparable damage occurs to the spindle apparatus following electrical stimulation. Indirect evidence from the present study suggests that, since the latter is not



associated with an increase in aneuploidy, the damage induced to the cytoskeletal elements that will subsequently reform to produce the spindle apparatus is probably only transient, readily repaired, and probably has no long-term detrimental effect on chromosome segregation.

The "spontaneous" rate of aneuploidy in one-cell fertilized embryos as observed at metaphase of the first cleavage division in this study was 0.6%, and this was not significantly different from the incidence observed with electrically-activated oocytes. The low rate of spontaneous aneuploidy in mice observed here is consistent with the findings of others (Donahue, 1972a; Röhrborn, 1972; Kaufman, 1973b; Dyban and Baranov, 1987), and is much lower than the estimates of aneuploidy reported to occur "spontaneously" in man where it has been conservatively estimated that at least 15-20% of all clinically recognised human pregnancies result in spontaneous abortion (Roth, 1963; Warburton and Fraser, 1964; Inhorn, 1967). Moreover, 50-60% of the latter are chromosomally abnormal (see, for example, Boué et al., 1975), with trisomics constituting the highest individual component group.

I observed that the activation rate of oocytes exposed to an electrical pulse in non-electrolyte solution was significantly greater than in the various control groups. This was not a postovulatory ageing effect, since all groups were matched to account for this factor. It is now widely accepted that it is not the electroactivation *per se* that activates the oocyte, but the influx of  $\text{Ca}^{2+}$  that occurs as a consequence of the electrical stimulus (Onodera and Tsunoda, 1989; Ozil, 1990; Fissore and Robl, 1992; Vitullo and Ozil, 1992; Rickords and White, 1992; Sun et al., 1992). Electroactivation is believed to create pores in lipid bilayers and make the membrane more permeable to chemical influx (Zimmermann and Vienken, 1982). Electroactivation of oocytes is only believed to occur in medium containing  $\text{Ca}^{2+}$  (Sun et al., 1992), but, in this study, electroactivation occurred in medium that was  $\text{Ca}^{2+}$ -free. However, if the oocytes remain permeable to extracellular  $\text{Ca}^{2+}$  for some minutes after activation (Rickords and White, 1992), washing in M16 medium in the presence of  $\text{Ca}^{2+}$ , as occurred in this study, would lead to a chemical influx and activation. It might be of interest to repeat this

experiment in the presence of aequorin to try to establish the exact sequence of events. Presumably, electroactivation in the presence of  $\text{Ca}^{2+}$  might be expected to lead to even higher rates of activation than observed in the present study (see Fissore and Robl, 1992; Rickords and White, 1992).

Only a small proportion of recently ovulated oocytes that were not given an electrical pulse but were, however, immersed in non-electrolyte solution for the same period of time as those in the experimental groups successfully activated. Activation in these cases may possibly have resulted from their exposure to  $\text{Ca}^{2+}$ -free medium which is in any case known to activate oocytes (Surani and Kaufman, 1977; Whittingham and Siracusa, 1978). It was also clear that handling the oocytes, and removing their surrounding cumulus cells with hyaluronidase, had an activating effect (Kaufman, 1973a; Kaufman and Surani, 1974), and the rates observed for oocytes electrically activated and previously exposed to these particular environmental conditions are likely, therefore, to have been cumulative.

It is now well documented that postovulatory aged oocytes activate more readily than recently ovulated oocytes (Kaufman, 1973a, 1983; Webb et al., 1986; Stice and Robl, 1988; O'Neill and Kaufman, 1988; Collas et al., 1989; Robl and Stice, 1989; Ware et al., 1989), but it is unclear whether this is also applicable in the case of human oocytes (Winston et al., 1991). It is important in cloning experiments, however, that recently ovulated oocytes are used, as postovulatory aged oocytes inevitably undergo postovulatory age-related detrimental changes, and are consequently likely to have a reduced long-term viability (Kaufman, 1983).

In the present study, I was able to activate approximately half of the oocytes in the hCG + 15.5 hours group, and it has been found that even very recently ovulated oocytes (ie hCG + 12 hours) can be activated following repetitive pulse stimulation (Ozil, 1990; Vitullo and Ozil, 1992). The reason why recently ovulated oocytes are apparently difficult to activate experimentally may be related to their response to the electrical pulse, since it has been suggested that intracellular  $\text{Ca}^{2+}$  levels do not change with ageing (Fissore and Robl, 1992). It

is thought that the level of stimulation employed (i.e. the number and strength of the pulses, and the field strength), and the "maturity" of the oocyte have an effect on the proportion of oocytes that are released from the meiotic "block" (Collas et al., 1989; Kubiak, 1989; Sun et al., 1992; Vitullo and Ozil, 1992). Kubiak (1989) reported that oocytes gradually develop the ability for full activation during the period of metaphase II arrest. He found that some oocytes, when subjected to an activating stimulus (provided by sperm or parthenogenetic agent), were able to resume anaphase movement and extrude a second polar body, but were unable to progress into the normal interphase state. These oocytes entered into a so called "metaphase III" phase. Cytogenetic analysis of these oocytes revealed the presence of unichromatid chromosomes. The latter oocytes could be reactivated after a period of a few hours, and this finding would seem to indicate that a certain "maturity" has to be achieved in order to complete meiosis. Strong parthenogenetic stimuli, such as heat or cold shock, can cause the activation of recently ovulated oocytes, but they also induce considerable damage to the cytoskeletal elements of the oocyte (Kaufman, 1983). In this study, recently ovulated oocytes lysed in greater numbers when subjected to a single electrical pulse than older oocytes, and this finding was also observed by others (see Collas et al., 1989).

Significantly more diploid and immediate cleavage parthenogenones were observed in electrically-activated oocytes than in the equivalent control groups. A similar situation is observed following the activation of increasingly postovulatory aged oocytes (Kaufman, 1973a, 1983), and this ageing effect was observed in both experimental and, to a lesser degree, the control groups of this study. The modification of the pathways that occurs as a consequence of ageing is most likely to result from the loss of the microfilament-rich area which overlies the spindle apparatus, and the "drifting" of the latter towards the centre of the egg, and thus the retention of both products of the second meiotic division within the egg (Szöllösi, 1971, 1975; Webb et al., 1986). The formation of immediate cleavage embryos has rarely been recorded in other studies involving the use of electroactivation (Onodera and Tsunoda, 1989; Ozil, 1990; Vitullo and Ozil, 1992). This may be the result of strain differences, but it is more likely that the type of parthenogene induced may either be dependent on the pulse duration given to

activate the oocytes or the character (ie the frequency and amplitude) of the pulse employed (Ozil, 1990), possibly through interference with the location or functioning of the cytoskeletal elements of the oocyte during the completion of meiosis.

I believe my findings have clearly demonstrated that the activation of oocytes by a single pulse electrical stimulation does not subject oocytes to a higher than baseline incidence of aneuploidy. Furthermore, the findings of others (see Vitullo and Ozil, 1992) would seem to suggest that repetitive pulse stimulation equally does not interfere with the normal events associated with chromosome segregation that occur at the time of activation.

## CHAPTER 9

### GENERAL DISCUSSION

## 9.1 THE FACTORS THAT INFLUENCE EARLY CLEAVAGE

Previous studies have revealed a number of factors which are known to influence early preimplantation development. For example, it is known that the timing of the first cleavage division in preimplantation mouse embryos is strain-dependent (McLaren and Bowman, 1973), and that the rate of cell division in early embryos is under genetic control (Goldbard and Warner, 1982). The rate of cell division in embryos is also known to be influenced by the environment (temperature, culture medium) (Chatot et al., 1989), since embryos cultured *in vitro* develop slower than those which develop *in vivo* (Bowman and McLaren, 1970). The poor, and often, abnormal growth and premature death of genetically abnormal embryos, both in humans and animals, revealed that there were other factors to uncover which might influence their early development. My examination of preimplantation polyploid embryos has enabled, firstly, the influence of ploidy and, secondly, the influence of the parental genomes on early cleavage to be investigated.

Firstly, my findings indicate that polyploidy *per se* does not confer a developmental disadvantage during the preimplantation period. The cleavage rate of triploid and tetraploid mouse embryos, during this time was similar to that of appropriately matched diploid embryos maintained under identical conditions. These results are valuable because in all previous studies of this nature, only rarely were attempts made to exclude the influence of some experimental variables, but in no cases were all variables excluded when comparisons were made between control and genetically abnormal material.

The morphological appearance of preimplantation polyploid embryos is also not dissimilar from that of diploid controls. Triploid mouse embryos, whether diandric or digynic in origin, contain approximately the same number of cells at the blastocyst stage as diploids. Similarly, homozygous tetraploids (which initiated development following electrofusion of blastomeres at the 2-cell stage, and consequently possess only half the cell number of diploids at this time) still possess approximately half the cell number of diploid blastocysts at the time of implantation. Triploid and tetraploid embryos compact and form a blastocoele at similar times

as diploid embryos after initiating development. An earlier and much cited study undertaken by Beatty and Fischberg (1951), which attempted to examine and summarise the influence of ploidy on cleavage, led these authors to hypothesise that "the number of cells in polyploid embryos of the same developmental age is inversely proportional to the number of chromosome sets present". This implied that the greater the amount of DNA in the nucleus, the longer it would take for the cell to replicate. My results demonstrate that such a relationship is, in fact, both simplistic and incorrect. It should be stressed that the hypothesis of Beatty and Fischberg (1951) was based on a very small sample of both spontaneously occurring and experimentally-induced polyploid material. They reported, for example, that tetraploid embryos had about half the cell number of that found in diploid embryos, but it was not apparent from their study whether the *rate* of tetraploid development was similar to that of diploids.

An interesting finding that emerged from my study, was that while the addition of one or more haploid sets of chromosomes does not appear to affect cleavage rate and rate of morphological development, the loss of a set of haploid chromosomes clearly does. In general, fewer haploid parthenogenones reached the blastocyst stage compared to diploid parthenogenones (see also Kaufman and Sachs, 1976). It is still unclear why this is the case, but it may be as a consequence of a deficiency in the normal diploid genome, since both human and mouse monosomic aneuploids also rarely survive beyond implantation (Epstein, 1986). Haploidy, for example, could result in decreased gene activity which may be detrimental for normal cleavage although, interestingly, a deficiency in enzyme activity by as much as 50% in heterozygotes is rarely accompanied by readily demonstrable metabolic changes (Kacser and Burns, 1973 (as reviewed in Epstein, 1986).

It has been pointed out, however, that haploid development improves when the nuclear-cytoplasmic ratio is altered to bring it closer to the diploid (presumably optimal) ratio (Kaufman and Sachs, 1975; Tarkowski, 1977; McGrath and Solter, 1986). This might indicate that a critical concentration of gene products is required for a specific cytoplasmic volume for



optimal cleavage to occur. If the synthesis of gene products continued at a dose-dependent rate in the cells of polyploid embryos, then normal development might be expected to occur. In parthenogenetic single-pronuclear haploid cells, however, the cytoplasmic volume is invariably doubled relative to the nuclear volume (when a comparison is made to the situation present in a normal diploid embryo) and such a critical concentration of gene products may not be reached in the same timescale as in diploid cells. This area would be interesting to examine further, since it may shed light on some of the requirements for cell division.

A variety of studies have shown that the presence of both parental genomes in the same cell, and in the appropriate ratio, is essential for embryonic development to term (Barton et al., 1984; Mann and Lovell-Badge, 1984; McGrath and Solter, 1984; Surani et al., 1984). Such studies have shown that the parental genomes have complementary roles during development, and that the maternal genome is believed to play a significant role in the development of the embryo proper (Surani et al., 1984; Surani, 1984, 1985), while the paternal genome is believed to direct the proliferation and development of the extra-embryonic tissues (Barton et al., 1984; 1985; Surani, 1985). The disparity in the roles of the parental genomes is the result of epigenetic modifications of some parental alleles which give rise to functional differences between certain homologous chromosomal regions (Cattanach and Kirk, 1985; Cattanach, 1986; Solter, 1988; Surani, 1991).

It was apparent from my results that an exclusively maternally-derived diploid genome, in parthenogenetic diploid embryos, was not detrimental to normal cleavage and the achievement of apparently morphologically normal stages of preimplantation development. Interestingly, however, it has been demonstrated that the development of androgenetic diploid embryos to the blastocyst stage is retarded (Surani et al., 1986; Latham and Solter, 1991; Mann and Stewart, 1991), and relatively few of these embryos reach the blastocyst stage compared to controls (Barton et al., 1984; Kaufman et al., 1989a). While this retardation can be partly attributed to the trauma associated with removing the female pronucleus (Modlinski, 1980), it is likely from these previous findings that an exclusively paternally-derived genome is

detrimental for preimplantation development. Furthermore, of those diploid androgenones which are capable of reaching the blastocyst stage, very few develop beyond the early postimplantation period. It is possible that there is a deficiency in one or more of their metabolic pathways, which may be critical for further development. The fact that they appear, almost selectively, to develop extra-embryonic rather than embryonic tissues may be a reflection of the fact that the embryonic and extra-embryonic tissues have different metabolic requirements during this period.

The roles of the parental genomes in development can also be examined in diandric and digynic triploid embryos. It was interesting to find that there was no difference in cleavage rate between these two classes of triploids during the preimplantation period, nor did they differ in their rate of development from the appropriate diploid fertilized controls. However, this result, together with the parthenogenetic information, strongly suggests that the presence of a paternally-derived haploid genome is not essential for early embryogenesis, while the presence of a maternal genome is. These findings lend weight to the current views on the complementary roles of the parental genomes on development, and particularly the critical role of the maternal genome. This is almost certainly the reason why a significantly greater proportion of diandric triploids compared to androgenetic diploids, can successfully complete early embryogenesis.

It has recently been observed in mice, that normal fertilized diploid female embryos are developmentally retarded (by a matter of hours) compared to males and to XO females when examined at 10.5 days of gestation (Thornhill and Burgoyne, 1992). This observation has also been noted during the preimplantation period (Tsunoda et al., 1985). The reason for the developmental retardation of diploid female compared to normal male embryos is believed to result from the influence of their paternally-derived X chromosome. This might explain the poor development of androgenones since they possess exclusively paternally-derived X chromosomes, and indeed in mice, XX diploid androgenones are never recovered after implantation while XY diploid androgenones are (Kaufman et al., 1989a). It is relevant to note

here, however, that no developmental/morphological differences exist between digynic XXX and XXY triploid embryos (Speirs and Kaufman, 1989b), or between diandric XXX and XXY triploid embryos on day 10 of gestation, but XYY embryos do appear to be developmentally retarded, since few of these embryos reach advanced somite stages (Kaufman et al., 1989c). It is unclear, therefore, whether the presence of one or two paternally-derived X chromosomes has any influence on diandric compared to digynic triploid preimplantation development. The use of male mice carrying a Robertsonian translocation involving the X chromosome, such as Rb(X.2)2Ad, in a similar experiment to the one presented in this thesis, might shed light on this question.

The specific influences that each parental genome has on development is readily demonstrable after implantation in the phenotypes of parthenogenetic, androgenetic and triploid embryos. The poor development of the trophoblast and the limited growth and development of parthenogenetic diploid embryos to the 25-somite stage on day 10 of gestation but not beyond this stage (Kaufman et al., 1977), would seem to confirm the essential role of the paternal genome in directing the development of the extra-embryonic tissues. The presence of two haploid maternal genomes, in addition to a paternal genome, in the cells of digynic triploid embryos also results in development (albeit usually abnormal) of the embryo proper, but this is often associated with poor development of the extra-embryonic tissues (Surani and Barton, 1983; Kaufman and Speirs, 1987; Kaufman et al., 1989b, Speirs and Kaufman, 1989a). The dominant influence of the two female genomes appears to outweigh the presence of the paternal genome, so that its influence is hardly detected. In contrast, the presence of two haploid paternally-derived genomes, in addition to a maternal genome, results in normal development of the extra-embryonic membranes. Furthermore, although a poorer development of the embryo proper would be expected (as the opposite phenotype of digynic triploids), diandric triploid embryos are, in fact, of similar size to digynic triploid embryos, and appear to be morphologically normal (Kaufman et al., 1989b, c). Finally, the absence of a maternally-derived genome results in androgenetic conceptuses which exhibit poor embryonic growth, so that these embryos rarely possess more than about 6-8 pairs of somites. However, they do

develop extensive trophoblastic tissue (Barton et al., 1984; Kaufman et al., 1989a). Clearly much more work has to be carried out on this and similar material before the relationship between the paternal and maternal genomes is established unequivocally.

## 9.2 WHY DO POLYPLOID MOUSE EMBRYOS DIE?

Triploidy and tetraploidy are lethal conditions in all laboratory animals. In humans, however, a few such embryos are able to survive to term. No consistent syndrome has been reported for the abnormalities found in triploid and tetraploid human abortuses and mouse embryos, though mouse tetraploids have a characteristic craniofacial appearance. Human triploid abortuses are commonly observed as well-formed embryos, but they are usually severely developmentally retarded. The majority of human tetraploid abortuses are usually found in the form of intact but empty gestational sacs, and very few embryos are recovered (Carr, 1971b; Warburton et al., 1991). In all strains of mice so far investigated, triploid embryos have never been reported to survive to term, it being usual for these embryos to die at about the limb-bud stage (for reviews, see Dyban and Baranov, 1987; Kaufman, 1991b). However, recently a live (possibly diandric) triploid mouse embryo was recovered on day 14 of gestation but was found to be developmentally retarded by about one day (Bos-Mikich and Whittingham, 1992). Some tetraploid mouse embryos have been reported to survive to term (Snow, 1973, 1975, 1976). This appears to be a very strain-dependent phenomenon since only the Q strain of mice used by Snow achieved development to term. Moreover, in subsequent studies by Snow, the earlier findings, even in the same strain, could not be repeated (M.H.Kaufman, personal communication). Tarkowski et al. (1977), for example, produced cytochalasin B-induced tetraploid mouse embryos from crosses between F1 (C57BL x CBA) females mated to A strain males. The most advanced development was found on day 8 of gestation, and the egg cylinders that were recovered were retarded in development. Electrofusion-induced tetraploid embryos from matings of the same F1 females to Rb(X.2)2Ad males also developed poorly. The six most advanced tetraploids in this series were early somite stage embryos, and the majority of implants only contained extra-embryonic membranes which were only occasionally associated with the presence of an embryonic vesicle (Webb et al., 1992). A better degree of success was

obtained from the use of Rb(1.3)1Bnr males (Kaufman and Webb, 1990), since viable tetraploid embryos have been recovered from these experiments on day 16 of gestation (Kaufman, 1991a, 1992a). Such strain-dependent tetraploid variability in viability is reminiscent of the "triploidy syndrome" described by Wróblewska (1978), where certain crosses of mice commonly result in triploid conceptuses in which only an empty gestational sac is formed, while in other strains and strain crosses, limb-bud stage development has been achieved.

Although the underlying causes responsible for abnormal polyploid development and their premature death still remain obscure, it is possible to speculate on some of the factors which might make a contribution to their eventual demise. For example, the rate of cellular metabolism is believed to be related to cellular volume. Since large cells have a small surface area/volume ratio, it has been suggested that the cell surface area may be a limiting factor in the cellular metabolism of large cells (Szarski, 1976). It is also possible that the poor development of polyploid embryos results from the fact that gene dosage expectations are not always met in polyploid cells. One expected consequence of polyploidy is a proportionate increase in the number of functioning genes in the genome and, if normal gene dose effects are operating, proportionate increases in the quantity of gene products might be expected to be produced. Although this has been reported to happen in tetraploid rat and human fibroblasts (Priest and Priest, 1969; Chang et al., 1983), Eglitis and Wiley (1981) reported that the activity of the enzyme, malate dehydrogenase, in the cells of tetraploid mouse embryos was significantly lower than the expected 2-fold increase. Furthermore, this was observed in embryos which had a cell number equivalent to that of diploid embryos (for review see Epstein, 1986). It is unclear whether the ability to regulate gene activity to the levels normally found in diploid cells may allow for more optimal development (Dyban and Baranov, 1987). This is clearly another area worthy of further investigation.



It is possible that the genetic background of polyploid embryos may also influence their survival, since in humans and mice, triploids with an XYY sex-chromosome constitution are rarely encountered (for refs. see Introduction). Human and mouse XXX and XXY triploids, whether diandric or digynic in origin, have a comparable developmental potential (although hydatidiform molar diandric triploid human conceptuses are reported on average to have a longer gestation, Jacobs et al., 1982). The situation is less clear with regard to tetraploidy, since a cytogenetic analysis of 120 human tetraploid abortuses revealed the sex-chromosome ratio to be 75 XXXX:45 XXYY (reviewed by Sheppard et al., 1982), while in homozygous tetraploid mouse embryos, most of which had achieved the 30 somite stage, the sex ratio was found to be 27 : 30, close to the predicted ratio of 1 XXXX:1XXYY (O'Neill et al., 1990).

It has been suggested that the number of inactivated-X chromosomes in human triploid embryos may have a significant effect on their developmental capacity (Jacobs et al., 1979). In a normal diploid cell, one of the two X-chromosomes present is inactivated to provide a mechanism of gene dosage compensation (Lyon, 1961). In the trophoctoderm and primitive endoderm of rodents, the inactivated X is usually paternal in origin (West et al., 1977; Frels et al., 1979), while in the embryonic ectoderm and extra-embryonic mesoderm, either the maternal or paternal X-chromosome may be randomly inactivated (Takagi and Sasaki, 1975; Wake et al., 1976). In LT/Sv strain digynic triploid mouse XXX embryos, however, there are two distinct cell lines present, one with two inactivated X-chromosomes and one with only one inactivated X-chromosome (Endo et al., 1982; Speirs et al., 1990). In yolk sac endodermal cells, the X-inactivation pattern always involves a paternally-derived X, unlike the random situation in the embryonic and mesodermally derived tissues (Speirs et al., 1990). Digynic triploid XXY mouse embryos of this strain also have two principal cell lines present, one with no X-inactivation and one with one X chromosome inactivated (Endo et al., 1982). Since no developmental advantage is gained by one particular triploid genotype in this strain of mouse (Speirs and Kaufman, 1989b), it is difficult to see why the X-inactivation status should affect the severity of the abnormalities in triploid mice. According to Jacobs et al. (1979), however, the embryonic portion of triploid human abortuses tends to have two active X-chromosomes

while the extra-embryonic membranes tend to have one active X-chromosome. The normal diploid ratio of autosomal loci to active X-chromosome loci is 2:1, and triploid embryos cannot achieve this, considering their X-inactivation patterns. It is still not clear which, if any, of the abnormal autosomal:active X ratios is the most detrimental to viability, but it has been proposed that the presence of two active X-chromosomes in the embryo (a ratio of 3:2) is more compatible with development, and that this may explain why human triploids occasionally reach term.

Snow (1975) suggested that an abnormal X-inactivation pattern may account for some of the developmental problems encountered in tetraploids. Several patterns of X-inactivation in the tissues of experimentally-induced homozygous XXXX tetraploid mouse embryos are encountered, but the majority of tissues show only two inactivated X-chromosomes, with preferential paternal X-inactivation in endodermally-derived tissue of the yolk sac (Webb et al., 1992). If one active X per two sets of autosomes is optimal, then the majority of tetraploids achieve this optimal balance.

I have demonstrated that the majority of polyploid mouse embryos do not die during the preimplantation development, and that their potential for further development beyond the blastocyst stage is reasonable, and should be on *a priori* grounds comparable to that of diploid fertilized embryos, since they are not developmentally retarded (at least in terms of cell number and morphological status) at the time of implantation. Therefore, we are able to exclude developmental retardation during the preimplantation period as a factor contributing to their poor viability.

My results have also demonstrated that an increase in ploidy has an effect on the cellular and nuclear volume and density of cells within the tissues and organs of postimplantation mouse embryos. Furthermore, all of the tissues and organs analysed in triploid and tetraploid embryos



demonstrated this effect and so this is probably unlikely to be restricted to a few specific cell types. It is possible that part of the reason for the abnormal development of triploid and tetraploid embryos may be geometrical in nature, as discussed below.

### *Tetraploidy*

My results showed that the cell and nuclear volumes of primitive red blood cells were increased in a predictable way in tetraploid embryos compared to those of diploid controls. Similarly, the nuclear volumes of the cells of the neural tube, the heart and two types of mesenchyme were also increased in tetraploid embryos compared to those of controls. Despite this increase in the size of the cells, the crown-rump lengths of tetraploid embryos were (and have been found to be) invariably smaller than those of developmentally matched diploid embryos. This infers that either the cells within the tissues and organs of polyploid embryos are more densely packed than in diploids, or that there is a reduction in the total number of cells present within these tissues and organs compared to diploids. An analysis of advanced postimplantation tetraploid embryos revealed that there was only about 40% of the cells found in developmentally matched diploid embryos, but since implanting tetraploid blastocysts have only about half the cell number of diploid blastocysts, no significant reduction in the expected number of cells present in these embryos occurred. Instead, the organs and tissues of these advanced tetraploids contained cells which appeared to be quite loosely packed together in a manner which would maintain the normal diploid size of the tissue or organ. A clear example of this was found in the neural tube where the cross-sectional areas of this tissue were found to be about the same in tetraploids as in developmentally-matched diploids. Clearly, there are size regulation mechanisms which are operating to maintain normality within the tetraploid embryo. Size regulation has been shown to occur for perturbations in cell number (e.g. in aggregation chimeric embryos), but has not so far been demonstrated for cell size (Tarkowski, 1963; Mintz, 1971; Buehr and McLaren, 1974; Lewis and Rossant, 1982; Rands, 1986a). My morphometric analysis of polyploid embryos has demonstrated that size regulation mechanisms take into account both cell number and cell size in order to prevent embryos being of abnormal proportions.

The morphology of the tissues and organs described above may explain some of the developmental problems encountered in tetraploids at least, as has also been suggested by Snow (1975). The most consistent abnormalities in tetraploid mouse embryos are found in the brain, and in particular the forebrain (Kaufman and Webb, 1990). Here, the abnormalities range from partial to incomplete separation of the telencephalic hemispheres, and other abnormalities involving the eyes and pituitary have also been described (also Snow, 1975). I have proposed that these structural abnormalities result from the presence of an inadequate number of cells, particularly in the forebrain, for the necessary functions to be separately distributed to the tissues by the underlying pattern-formation mechanisms. Similar abnormalities involving the forebrain were reported by Snow (1975), and it should be noted that some tetraploid mouse embryos reported by Snow were compatible with live birth (Snow, 1973, 1975) though it is likely that these embryos were in fact diploid-tetraploid mosaics rather than "pure" homozygous tetraploids. The postcranial morphological features of individual tetraploid mouse embryos have been reported to show at least one major congenital abnormality, involving at least one organ system, but these were not considered to be life-threatening (Kaufman, 1992a). It is probable that the geometrical problems encountered by the tetraploids result in the abnormalities described for these embryos. It seems unlikely, however, that these abnormalities are always the cause of the premature death in tetraploid embryos.

### *Triploidy*

The morphological differences and abnormalities observed between digynic and diandric triploid mouse embryos appear not to arise from geometrical problems since the number and size of their constituent cells are identical. Cell and nuclear volume in triploid embryos is increased to nearly 1.5 times the size of similar measurements made on developmentally-matched diploid cells. In other species such as amphibia and fish, triploidy also results in an increase in the size of the component cells, and a concomitant reduction in cell number prevents the embryo from being of giant proportions (Fankhauser, 1941, 1945; Small and Benfrey, 1987). Although cell density in triploid tissues and organs was not analysed, it is probable that a similar size regulation mechanism occurs in triploid mammalian embryos. This

mechanism is likely to occur shortly after implantation, since triploid blastocysts have a similar number of cells as diploid blastocysts yet no postimplantation mouse triploids recovered to date have been reported to be unusually large. Furthermore, this is also believed to be the time during development when size regulation occurs in aggregation chimeric embryos.

The inconsistent phenotypes of postimplantation diandric and digynic triploid embryos are more likely to arise from imprinting effects. An abnormal dosage of maternally or paternally imprinted genes may be involved in the disrupting of positional information required for normal tissue formation resulting in the spectrum of abnormalities seen (Barton et al., 1991). The most consistent features of diandric and digynic triploid mouse embryos are general retardation (in both types) and craniofacial and neural tube abnormalities (principally confined to the digynic group). Diandric and digynic triploid embryos, however, both die at around midgestation and therefore these abnormalities do not seem to selectively disadvantage the viability of one or other group.

In general terms, the geometrical problems associated with triploidy should be less severe than that of tetraploidy, since the size of triploid cells are smaller than tetraploid cells and hence, the resultant perturbation in tissue and organ structure associated with cellular density will be of a smaller magnitude. However, triploid mouse embryos consistently die at an earlier stage in gestation than tetraploids and so other explanations must be sought. The death of triploids is likely to be related to the fact that triploid embryos, unlike tetraploid embryos, are invariably genetically unbalanced. Cattanach and Kirk (1985) showed that disomy/nullisomy for certain autosomes in the mouse genome resulted in varying phenotypes depending on the parental origin of the autosomes. Such regions of these autosomes have been subject to a germ line imprint which renders the maternal and paternal copies functionally dissimilar in development. It is thought that the reciprocal activity (i.e. complementarity) and functional hemizygosity of imprinted parental alleles are utilised to co-ordinate embryonic growth, cell proliferation and the development of specific cell lineages. This may occur through the regulation of the dosage

of genes involved in these processes (Surani et al., 1990). An influence of imprinted genes on the development of specific cell lineages is clearly seen in chimerics formed from androgenetic and fertilized embryos and parthenogenetic/gynogenetic and fertilized embryos. Androgenetic cells in these chimeras contribute mainly to muscle, skeleton and heart (Mann et al., 1990; Barton et al., 1991), while parthenogenetic/gynogenetic cells in chimeras make greater contributions to other organs such as the brain and germ cells (Stevens et al., 1977; Surani et al., 1977; Stevens, 1978; Anderegg and Markert, 1986; Fundele et al., 1989, 1990; Nagy et al., 1987, 1989). Abnormal gene regulation, resulting from a doubling of one or other parental genome (in addition to the normal genome) could conceivably result in the abnormal development of some cell lineages in triploid embryos (e.g. neural tissues), the direct or indirect consequences of which might be life-threatening.

Homozygous tetraploid embryos are not genetically unbalanced and so their abnormal development might not occur through such obvious aberrations in gene regulation. Tetraploid embryos, however, have to cope with greater geometrical problems than triploid embryos but clearly some of these embryos are able to cope with these morphological abnormalities well into the postimplantation period. The premature death of tetraploid mouse embryos which are able to cope with these developmental problems may simply be a consequence of inadequate functioning of the placenta since it apparently shows certain histopathological features consistent with this hypothesis. Shunting of the blood cells within the superficial tissues of the placenta has been observed (M.H. Kaufman, personal communication) which would lead to poor oxygenation and nutrient supply to the embryo. The latter, however, is not relevant to the triploids since they do not reach the stage of development when a normal placenta is present.

The reasons for the premature death and abnormalities in human polyploids are likely to be more complicated. Placental abnormalities are likely to contribute to the death of human triploid embryos since it is known that about 85% of human triploids are diandric, and this is associated with the development of the "partial" hydatidiform mole (Jacobs et al., 1982). The degenerative changes within the placenta of partial moles may lead to death of the embryo

through inadequate placental function. No consistent histopathological features have so far been described in relation to digynic triploid embryos, but their death is likely to be related to their genomic imbalance. Human tetraploids are rarer, with only a few reaching term, while the majority abort as empty gestational sacs. The reason why some tetraploid embryos should survive while others do not may reflect their genetic status. It is unclear whether homozygosity is more lethal than heterozygosity in polyploid development. A survey of 120 human tetraploid spontaneous abortions reported only the ratio of XXYY and XXXX sex chromosome groups, which was found to be 45 : 75 (Sheppard et al., 1982), and the absence of any obvious heterozygotic groups would seem to suggest that they are the less viable class. However, it is possible that this data may not exclusively comprise of homozygotes. It is conceivable, however, that triandric tetraploidy may be more lethal than tetraploidy resulting from an error of cleavage (i.e. homozygous), since conceptuses of the former type have been associated with the presence of a "partial" hydatidiform mole (Sheppard et al., 1982; Surti et al., 1986).

It appears that the human genome, in contrast to other mammals, is able to cope better with an increase in ploidy since some polyploid embryos are able to reach term. It is worth considering finally, however, that this may not be genetical but rather a result of the fact that many more human fetuses have been examined compared to those of other species. Perhaps if the number of experimental animals and their offspring examined were to be increased, polyploid development to term might occasionally be observed.

### 9.3 METHODOLOGICAL CONSIDERATIONS

The results of previous studies on the effect of parthenogenetic stimuli on the incidence of aneuploidy in parthenogenones, highlighted the need for a similar examination to be carried out on electroactivation as a parthenogenetic stimulus. The results were reassuring, as ovulated oocytes activated by a single electrical pulse did not exhibit a significantly higher incidence of aneuploidy than that found in unstimulated controls. This demonstrated, therefore, that this parthenogenetic stimulus was not detrimental to the spindle or spindle precursors.



Electrical pulse stimulation is very efficient, particularly at activating very recently ovulated oocytes, an important consideration in cloning experiments because aged oocytes are believed to be in a state of degeneration and are consequently of "poorer quality" (Collas et al., 1989). In addition, because repetitive pulse stimulation is able to simulate  $\text{Ca}^{2+}$  oscillations, which are known to occur at fertilization, its potential uses may include the examination of the precise role of these oscillations in the initiation of the development of an embryo. Ozil (1990), for example, has demonstrated that some developmental stages such as compaction and blastocyst formation in parthenogenetic embryos can be influenced by the process of egg activation some days earlier. I believe that improvements in the electroactivation technique and the activation of very recently ovulated oocytes will eventually enable the investigation of mouse parthenogenetic development under more optimal conditions than are used at the present time. Furthermore, these advances in methodology may also facilitate the postimplantation development of parthenogenones beyond the forelimb bud stage, which is the most advanced stage so far achieved by these embryos (Kaufman et al., 1977; Kaufman, 1983).

#### 9.4 CONCLUSIONS

For successful normal development to term, the mouse embryo must have a diploid genome which contains both a maternally and paternally-derived set of chromosomes. However, duplication of one or both parental genomes leading to the triploid or tetraploid status respectively, still allows apparently normal preimplantation development to occur, similar to that of fertilized diploid embryos. Single-pronuclear haploid parthenogenones, however, have a slower cleavage rate compared to that of diploid parthenogenones, and their potential for further development is undoubtedly limited as a result. It appears, therefore, that the addition of "extra" haploid genomes to a diploid genome is not detrimental to early development, while the loss of a haploid genome, without a concomitant reduction in cytoplasmic volume, undoubtedly is.

My results have confirmed that the presence of a maternal genome is important for normal early embryogenesis. I showed that diploid parthenogenones and diandric and digynic triploid embryos were able to develop as well as fertilized diploid embryos during their preimplantation development, and others have shown that diploid androgenones (which contain an exclusively paternally-derived genome) apparently do not develop as well during this time. There were no genomic imprinting differences between diandric and digynic triploid embryos with regard to cell number, morphological appearance before implantation or cellular morphology.

A polyploid genome always results in abnormal development and premature death of embryos after implantation. Since the preimplantation development of these embryos is apparently normal, their developmental failure cannot be attributed to poor cellular proliferation during this early stage of their development. All triploid mouse embryos die at around midgestation, while some tetraploid embryos have been reported to have reached term, but the majority die at earlier stages of development.

A relationship was established between cell and nuclear volume and ploidy. Polyploid embryos, however, are not excessively large due to the presence of larger cells, in fact, their body size is usually smaller than that of diploids. To maintain their relatively normal size, tetraploid embryos have a significantly lower cell number than that found in diploid embryos, and this may partly account for the abnormal development of their tissues and organs. However, this is unlikely to result in the death of all tetraploid embryos. Their premature death may simply be attributed to a poorly developed placenta. In the case of triploid mouse embryos, it is likely that the geometrical problems associated with polyploidy (i.e. increased cell volume, low cell number) are probably not as important as the fact that triploid embryos are genetically unbalanced. Strain and species differences are also likely to complicate the understanding of the pathology of polyploid embryos.



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# Cleavage Rates of Diploid and Tetraploid Mouse Embryos During the Preimplantation Period

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**ABSTRACT** Despite the fact that spontaneous tetraploidy is a rare phenomenon in mice, such embryos may be produced experimentally by a variety of means, though only a very limited degree of postimplantation development has been achieved. Despite this apparent limitation, much data on the rate of development of preimplantation tetraploid embryos has been published. However, the findings from these studies has often been conflicting. In the light of the recent successful achievement of advanced postimplantation tetraploid development in our laboratory, we decided it was an opportune time to re-evaluate the preimplantation development of these embryos in as near to optimal conditions as we could achieve.

Three groups were studied, namely 1) control (diploid) embryos developing *in vivo*, 2) control (diploid) embryos that had been isolated at the 2-cell stage, briefly retained in culture, then transferred to the oviducts of pseudopregnant recipients, and 3) tetraploid embryos produced by electrofusion of blastomeres at the 2-cell stage, then transferred to the oviducts of pseudopregnant recipients. Embryos were isolated from females from each group at specific times after the HCG injection to induce ovulation. The total cell number of each embryo was established and the log mean values were plotted against time. From the gradients of the lines it was possible to establish that there was a significant difference between the cell doubling time of the transferred controls (group 2) compared to the *in vivo* controls (group 1) with cell doubling times of  $15.86 \pm 1.45$  h and  $10.27 \pm 0.24$  h, respectively. However, there was no significant difference between the cell doubling time of the tetraploids and the transferred controls (group 2), with the tetraploids having a cell doubling time of  $14.87 \pm 0.54$  h. The results therefore show that under matched experimental conditions, tetraploid mouse embryos develop during the preimplantation period at the same rate as diploids despite their reduced cell number. This finding suggests that the various hypotheses that have been proposed to explain the poor postimplantation development of tetraploids, related principally to their supposed poorer proliferative activity than controls, does not appear to be applicable at least in relation to their preimplantation development.

Studies on the timing of the events that occur during the preimplantation period are capable of shedding important light on the factors necessary for successful blastocyst formation and postimplantation development.

Embryonic cell number is a useful parameter for establishing, for example, the optimum culture conditions for preimplantation embryos (Bowman and McLaren, '70) or for determining, albeit indirectly, when blastocyst formation occurs (Smith and McLaren, '77; Eglitis and Wiley, '81). It is likely therefore that cell number may also be useful when analysing polyploid mammalian embryos in order to investigate the morphogenetic changes that occur during the pre- and early postimplantation period that may be a direct consequence of their genetic constitution.

We have recently become interested in the in-

fluence of ploidy on early embryonic development. Despite the fact that spontaneous tetraploidy is a rare phenomenon in mice (Beatty, '57; Dyban and Baranov, '87), such embryos may be produced experimentally by a variety of means (see, for example, Snow, '73; '75; O'Neill et al., '90; Kaufman and Webb, '90). When tetraploid embryos are produced by electrofusion (Kaufman and Webb, '90), the increase in ploidy in these homozygous tetraploids is due to their having twice the normal chromosome complement. The embryos therefore possess two identical maternal and two identical paternal genetic contributions.

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Despite the fact that a wide variety of techniques has been used to induce tetraploid development, the successful birth of liveborn tetraploids has only very rarely been reported. Thus Beatty and Fischberg ('51), for example, induced the development of preimplantation tetraploids in mice by circulating hot water around the oviduct at the estimated time of first cleavage. Colchicine induced suppression of the first cleavage has also been used with some success (Edwards, '58a). Other agents such as inactivated Sendai virus (Graham, '71; O'Neill et al., '90) and polyethylene glycol (Eglitis, '80; Eglitis and Wiley, '81; Spindle, '81), that have been employed to induce tetraploidy, have also resulted in only a very limited degree of postimplantation development. Snow ('73, '75, '76) was more successful, with the exposure of 2-cell stage mouse embryos to cytochalasin B, which inhibits cytokinesis but not karyokinesis. Unfortunately, to date, no others have reported development beyond implantation using this technique. Despite this apparent limitation, much data on the rate of development of preimplantation tetraploid material has been accumulated using this means of producing tetraploid embryos (Tarkowski et al., '77; Smith and McLaren, '77). In one of these studies (Smith and McLaren, '77) the preimplantation development in vitro of cytochalasin B-induced tetraploid embryos was compared with that of control diploid embryos. These authors observed that the mean cell number of cytochalasin-induced tetraploid blastocysts was slightly less than half of that of control diploids incubated under similar conditions. A significant proportion (over 40%) of their tetraploid embryos, and a somewhat smaller proportion (18%) of their controls, however, had died by 60 h incubation in vitro. Any effect of ploidy in this experimental system therefore may have been accentuated by the less than optimum culture conditions employed by these and other researchers. Because in vitro culture conditions are inevitably suboptimal compared to conditions in vivo (see, for example, Bowman and McLaren, '70; Streffer et al., '80; Harlow and Quinn, '82), any attempt at comparing the influence of ploidy on development should, where possible, we believe, take this factor into account.

Recently, electrofusion has been shown to be an extremely efficient fusigenic stimulus, and has allowed high rates of early postimplantation development of homozygous tetraploidy to be achieved (Kaufman and Webb, '90). This experimental approach has previously been applied to

rabbit embryos (Ozil and Modliński, '86), bovine embryos (Iwasaki et al., '89), as well as to mouse embryos (Kubiak and Tarkowski, '85; Kato and Tsunoda, '87; Winkel and Nuccitelli, '89), though in these studies, only preimplantation development was successfully achieved. These, but more particularly our own recent studies (Kaufman and Webb, '90), would seem to indicate that electrofusion per se has little or no obvious deleterious effects on preimplantation development, unlike other fusigenic agents, despite the fact that only a limited degree of postimplantation development has so far been reported.

In the light of the recent successful achievement of postimplantation tetraploid development in our laboratory (Kaufman and Webb, '90), we decided it was an opportune time to re-evaluate the preimplantation development of these embryos in as near to optimal conditions as we could achieve. In particular, we were interested to establish any effect of ploidy on cleavage rate during the preimplantation period, as it has been reported that the cleavage rate of digynic triploids over this period is significantly slower than that of diploids (Takagi and Sasaki, '76). However, in triploidy, it is far more difficult to establish whether any effect observed is a consequence of ploidy per se, or is due to the effect of a genetically "unbalanced" genome.

We also wished to demonstrate that experimental procedures, even the isolation of embryos, followed by their brief incubation in vitro followed by their rapid return to an appropriate recipient, can have a significant effect on the findings.

## MATERIALS AND METHODS

### *Control series 1*

Eight- to twelve-week-old (C57BL × CBA)F1 hybrid female mice were injected with 5IU pregnant mares' serum gonadotrophin (PMSG) followed 48 hours later with 5IU human chorionic gonadotrophin (HCG) to induce ovulation. After the HCG injection the females were caged individually with fertile homozygous Rb(1.3)1Bnr male mice. The presence of a vaginal plug the next morning was taken as evidence of mating and this was considered to be the first day of gestation. At specified time points after the HCG injection, the female mice were sacrificed by cervical dislocation and the oviducts or uteri removed and flushed through with Dulbecco's phosphate buffered saline (PBS) containing 4% bovine serum albumin (BSA) in order to recover the em-



bryos. Air-dried preparations were made of these embryos using a modification of the technique described by Tarkowski (Tarkowski, '66) and the preparations were stained with 10% giemsa to enable cell counts to be made.

### **Control series 2**

F1 hybrid female mice were superovulated and mated with homozygous Rb(1.3)1Bnr males as indicated above. Early on the morning of the second day of gestation, the female mice were sacrificed by cervical dislocation and the oviducts removed and flushed through with PBS containing 4% BSA in order to recover the 2-cell stage embryos. These embryos were then transferred into drops of tissue culture medium (Whittingham, '71) under paraffin oil, and retained for 2–3 hours in an incubator maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> in air.

These 2-cell stage embryos were then transferred unilaterally to the oviducts of recipients on the first day of pseudopregnancy (i.e., on the day of finding a vaginal plug after spontaneously cycling females had been mated to proven sterile vasectomised males). The recipients were anaesthetised with tribromoethanol (Avertin, Winthrop, dose 0.02 ml/g body weight of a freshly prepared 1.2% solution of Avertin dissolved in 0.9% saline). At specified time points after the transfers, the recipient mice were sacrificed and the operated oviducts or uteri removed and flushed through with PBS containing 4% BSA in order to recover the transferred embryos. Air-dried preparations were made of these embryos and cell counts made as described above.

### **Tetraploid series**

Two-cell stage embryos were recovered as described for the control series 2 above, and incubated in drops of tissue culture medium (Whittingham, '71) under paraffin oil at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. The 2-cell stage embryos whose blastomeres were to be fused were transferred into a non-electrolyte solution consisting of 0.3 M Mannitol (Kubiak and Tarkowski, '85) and a similar solution was also present in the fusion chamber. The latter consisted of a plastic tissue culture dish which had two platinum wires each of 250 µm diameter fixed parallel to each other on the bottom of the dish with a space of about 600 µm between them. The ends of the platinum wires were connected to a digitimer pulse stimulator set at 200 v, with a pulse duration of 50 µsec. The 2-cell stage embryos, in batches of

10, were then placed between the two platinum wires in the chamber and the pulse stimulator was triggered. The embryos were removed immediately and washed through 4 drops of tissue culture medium and then returned to the incubator. Within 15–30 mins the two blastomeres had fused in a very high proportion of cases to form a single blastomere. These 1-cell (tetraploid) embryos were then transferred unilaterally to the oviducts of recipients on the first day of pseudopregnancy, as described above.

At specific time points after the transfers, the recipients were sacrificed by cervical dislocation and the operated oviducts or uteri removed and flushed with PBS containing 4% BSA in order to recover the embryos. All cell counts were made as described above for the control series. In preliminary experiments these embryos were incubated for 3–4 h in the presence of colcemid to establish their ploidy. In all instances, the electrofused embryos were found to have a tetraploid chromosome constitution, so that in the definitive series air-dried preparations were made as soon as possible after the embryos were recovered. In all instances, note was made of the gross morphological appearance of individual embryos, such as whether they showed evidence of compaction, were clearly compacted, were at the early or late blastocyst stage, and whether they were zona-intact, or had escaped from the zona. Note was also taken as to whether the embryos were recovered from the oviduct and/or uterus.

## **RESULTS**

The findings from this study may most conveniently be considered first in relation to the total cell count of diploid (control) embryos, and electrofusion-induced tetraploid embryos at specific times during the preimplantation period, and secondly in relation to the gross morphological appearance of these embryos at these times.

### ***A comparison between the total cell count of diploid (control) embryos developing in vivo, and diploid (control) embryos that had been isolated at the 2-cell stage and returned after a brief incubation period in vitro to the oviducts of pseudopregnant recipients***

#### **In vivo findings**

When the mean value for the total cell count of embryos from the first category was established at various intervals after the HCG injection for inducing ovulation (see Table 1), these values

TABLE 1. Mean cell number of embryos recovered at various times after HCG injection to induce ovulation in control (*in vivo*) series 1, control (transfer) series 2, tetraploids series 3

| Group                                     | Time of isolation<br>(hours post HCG) | No. of embryos<br>transferred | No. recovered<br>(healthy) | No. analysed<br>to establish<br>cell number | Mean cell<br>number<br>( $\pm$ SEM) |
|---|---------------------------------------|-------------------------------|----------------------------|---|-------------------------------------|
| Control<br>( <i>in vivo</i> )<br>series 1 | 49                                    | N.A.                          | 80                         | 80  | $2.25 \pm 0.07$                     |
|   | 68                                    | N.A.                          | 56                         | 56  | $8.50 \pm 0.27$                     |
|   | 72                                    | N.A.                          | 57                         | 57  | $11.72 \pm 0.58$                    |
|   | 79                                    | N.A.                          | 78                         | 78  | $19.21 \pm 0.72$                    |
|   | 96                                    | N.A.                          | 44                         | 44  | $54.14 \pm 2.49$                    |
|   | 102                                   | N.A.                          | 39                         | 39  | $78.26 \pm 2.52$                    |
| Control<br>(transfer)<br>series 2         | 70                                    | 64                            | 56                         | 43  | $9.28 \pm 0.49$                     |
|   | 82                                    | 106                           | 61                         | 55  | $18.09 \pm 0.70$                    |
|   | 95                                    | 66                            | 52                         | 43  | $31.58 \pm 1.25$                    |
|   | 100                                   | 60                            | 51                         | 45  | $50.40 \pm 2.18$                    |
|   | 110                                   | 72                            | 36                         | 22  | $68.50 \pm 3.30$                    |
|   | 122                                   | 155                           | 88                         | 54  | $87.91 \pm 2.39$                    |
| Tetraploids<br>series 3                   | 74                                    | 53                            | 51                         | 48  | $4.69 \pm 0.24$                     |
|   | 81                                    | 61                            | 58                         | 52  | $7.31 \pm 0.20$                     |
|   | 97                                    | 63                            | 51                         | 38  | $15.21 \pm 0.46$                    |
|   | 105                                   | 93                            | 72                         | 56  | $21.68 \pm 0.83$                    |
|   | 116                                   | 111                           | 51                         | 37  | $33.54 \pm 1.80$                    |

were converted into natural logarithms and the latter were then plotted graphically against time after HCG injection (see Fig. 1). A line of best fit was obtained and this allowed a cell doubling time (in hours) to be determined (see Fig. 1). The cell doubling time ( $\pm$  S.E.M.) between 48 h and 100 h after the HCG injection was found to be  $10.27 \pm 0.24$  h.

#### Cell doubling time after isolation and brief culture of diploid (control) embryos at the 2-cell stage and their subsequent transfer to pseudopregnant recipients

When embryos from this group were isolated from the recipients at specific times after the original HCG injection to induce their ovulation, their mean total cell count was established as in group 1 above (see Table 1). These values were then converted into natural logarithms and the latter were then plotted graphically against time after HCG injection. A line of best fit was then obtained, and this allowed a cell doubling time (in hours) to be determined, in this series, between 72 h and 120 h after the HCG injection (see Fig. 1). The slope of the calculated line of best fit was found to be significantly different from the value obtained from the *in vivo* study, with in this case a cell doubling time ( $\pm$  S.E.M.) over the period studied of  $15.86 \pm 1.45$  h.

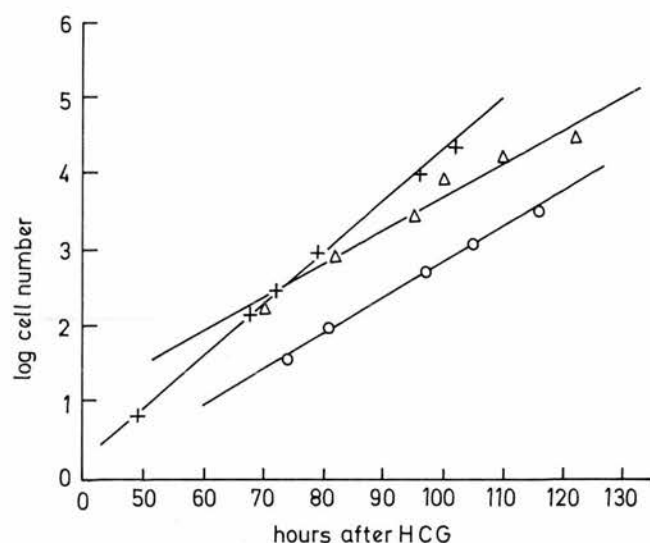


Fig. 1. Graph of log cell number of *in vivo* controls (1), control (transferred) embryos (2), and tetraploid (transferred) embryos (3) plotted against time after HCG injection to induce ovulation. By regressing the log (cell number) against time, it was possible to estimate the gradient of each line using least squares. The regression was weighted by the sample size for each mean value. From the gradient it was possible to estimate the average cell doubling time for each series, namely  $10.27 \pm 0.24$  h for series 1,  $15.86 \pm 1.45$  h for series 2, and  $14.87 \pm 0.54$  h for series 3. No significant difference was found between the cell doubling times of series 2 and 3, but the cell doubling time between series 1 and 2 was significantly different. Key: series 1, +; series 2,  $\Delta$ ; series 3,  $\circ$ .

**Cell doubling time of electrofusion-induced tetraploid embryos isolated from pseudopregnant recipients at various times after the original HCG injection for inducing ovulation**

This group was in all respects identical to group 2 except that the embryos were exposed to the electrofusion stimulus during their brief period of incubation in vitro. As in series 1 and 2 above, the mean total cell counts of the tetraploids was established at intervals after the original HCG injection (see Table 1). The values obtained were then converted into natural logarithms and plotted graphically against time after HCG injection. A line of best fit was obtained and this allowed a cell doubling time (in hours) to be determined, in this series between 75 and 115 h after the HCG injection (see Fig. 1). In this series, the calculated line of best fit was found to be not significantly different from the value obtained from series 2 above, with a mean cell doubling time ( $\pm$  S.E.M.) of  $14.87 \pm 0.54$  h. This value was also significantly different from the calculated mean doubling time for the embryos in the in vivo series.

***The location and morphological appearance of control and tetraploid embryos isolated at various times after their transfer to pseudopregnant recipients***

In both the control (series 2 above) and tetraploid series, embryos were almost exclusively located in the oviduct at all times up to 100–105 h after the original HCG injection. At 110 h in the control series, just over 70% of the embryos were still located in the oviduct, while just under 30% were recovered from the uterus. At 122 h, however, all of the embryos recovered were located in the uterus. In the case of the tetraploids, at 116 h after the HCG injection, over 90% of the healthy embryos recovered were located in the uterus.

In the control series, all of the healthy embryos isolated at 70 h after the HCG injection were compacted morulae (mean cell number ( $\pm$  S.E.M.)  $9.28 \pm 0.49$ ). The first blastocysts were recovered at 95 h after HCG, and constituted about 12% of the embryos recovered at this time. By 100 h after the HCG injection the blastocysts now constituted 74% of the embryos recovered. At 110 h, all the embryos recovered were at the blastocyst stage, and of these 31% were zona free, while at 122 h, 94% of the blastocysts recovered were zona free.

The situation with regard to the tetraploids was found to be remarkably similar, in that com-

pacting embryos (albeit at the 4-cell stage) were recovered at 74 h, and only fully compacted embryos at 81 h. By 97 h, 39% of the embryos recovered were at the blastocyst stage, while at 105 h, 83% were at the blastocyst stage, and of these only 5% were zona free. However, by 116 h, 83% of the blastocysts recovered from the uterus were zona free. Curiously, 3 blastocysts and a morula, out of a total of 51 healthy embryos isolated at this time, were recovered from the oviduct. It is apparent, therefore, from these findings that blastocyst formation and the time of achievement of the zona-free state was almost exactly the same as in the controls (See Fig. 2).

## DISCUSSION

Our results reveal that tetraploid mouse embryos cleave at approximately the same rate during the preimplantation period as diploids. A superficial analysis of cell number at particular stages of preimplantation development can imply this, as others have done. Thus Beatty and Fischberg ('51) found that the mean total cell number of six mouse tetraploids at 3.5 days of gestation was about half that of genetically similar control embryos. From this, and analyses of other classes of polyploid embryos, the authors hypothesised that "the mean number of nuclei in polyploid eggs of the same age is approximately in inverse proportion to the number of chromosome sets present." Analyses of mouse tetraploid blastocysts by others also revealed that the tetraploids contained about half the cell number of control blastocysts (Snow, '75, '76; Smith and McLaren, '77; Tarkowski et al., '77; Spindle, '81; Kubiak and Tarkowski, '85).

It has been suggested that this reduced cell number might possibly have resulted from their suppressed first (or second) cleavage division, since their *rate* of development appeared to be similar to that of diploids. The tetraploids in our study compacted at the same time as diploids (i.e., when they possessed about 4 blastomeres, equivalent to about the 8-cell stage in diploid controls) (see Kubiak and Tarkowski, '85; Kato and Tsunoda, '87) and formed blastocysts at the same time after fertilisation as controls (Smith and McLaren, '77; Eglitis and Wiley, '81). It is of interest to note that in our study, fully expanded blastocysts were flushed out from the oviduct at 95–105 h in both the control and tetraploid series. This is clearly due to the fact that these embryos were transferred to asynchronous recipients that were approximately 24 h earlier in gestation than



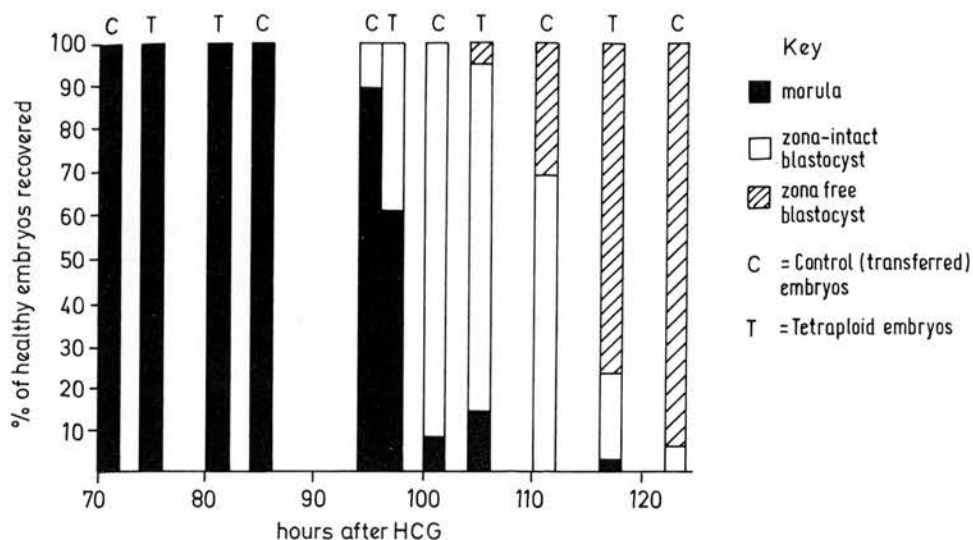


Fig. 2. The morphological appearance of diploid control and tetraploid embryos isolated at various times after the HCG injection to induce ovulation. In both the control and the tetraploid series, normal diploid embryos were isolated at the 2-cell stage and retained in culture for 2–3 hours. Control

embryos, and electrofusion-induced tetraploids, were then transferred to the oviducts of pseudopregnant recipients. The recipients were autopsied at various times after the HCG injection and embryos recovered from either the oviduct and/or uterus.

were the transferred embryos. By 116 h after the HCG injection virtually all of the control as well as the tetraploid embryos were zona free and found in the uterus.

Others have reported that the rate of development in octoploid mouse embryos is also similar to that of controls (Winkel and Nuccitelli, '89), thus demonstrating that ploidy per se (at least initially) does not appear to affect the developmental progress of preimplantation tetraploid or octoploid mouse embryos.

A reduced cell number also appears not to be detrimental to embryonic development. Tarkowski ('59) obtained normal liveborn young following the transfer to appropriate recipients of "half" blastocysts (i.e., embryos where one blastomere at the 2-cell stage was destroyed), and good post-implantation development has recently been obtained in our laboratory from tetraploid mouse blastocysts which presumably possessed half the cell number of diploid controls. Even with a reduced inner cell mass (ICM) cell number, which occurs in consequence of a reduction in total blastocyst cell number (Tarkowski and Wróblewska, '67), embryonic development still appears to proceed normally. No attempt was made here to establish ICM number, but unlike the tetraploids examined by Snow ('73, '75), all of the tetraploid blastocysts analysed in this study had an obvious ICM. Our recent results indicate that tetraploid blastocysts, even with their reduced total cell

number, have a similar expectation of developing into the early postimplantation period as diploids (Kaufman and Webb, '90), though admittedly their more advanced development is restricted.

Conflicting findings have been published regarding the cleavage rates of haploid and triploid mouse embryos. Preimplantation parthenogenetically activated haploid embryos apparently develop more slowly than diploids (Witkowska, '73; Kaufman and Sachs, '76). Similar findings have been reported with respect to gynogenetic haploids (Modliński, '75). Triploid mouse embryos, according to Edwards ('58b), cleave at a similar rate to diploids, while Takagi and Sasaki ('76) calculated that the cell cycle time in their digynic triploid mouse embryos was approximately 10% longer than in diploids.

What is also clearly evident from our results is the detrimental effect that the experimental handling of embryos has on their subsequent cleavage rate. Our *in vivo* findings are in close agreement with previous studies, with a preimplantation cell doubling time of approximately 10 h (Bowman and McLaren, '70; Allen and McLaren, '71; Barlow et al., '72; Streffer et al., '80; Harlow and Quinn, '82; Molls et al., '83). Estimates for cell doubling time for *in vitro* studies are more varied, possibly because of variability in the culture conditions employed. Bowman and McLaren ('70), for example, give a cell doubling time of about 24 h, Harlow and Quinn ('82) 17 h,



while Streffer et al. ('80) give 12 h. While it is not possible to make an exact comparison between our findings and other studies, it is interesting to note that in our study the cell cycle time of 2-cell stage embryos that had been isolated and subsequently transferred after a brief incubation period in culture to pseudopregnant recipients was approximately halfway between the calculated mean doubling time in vivo and the published figures for cell doubling time in vitro. Furthermore, our findings do not appear to show that the cell cycle time in the tetraploids varied during the period studied. Indeed, it was almost identical in all respects to that of the matched controls. This latter group, therefore, would seem to be a more meaningful control for the type of study undertaken here, than comparing the cell cycle time of the tetraploids with that of controls in vivo.

Although an initial slowing of cleavage appears to occur immediately after the transfer procedure, and indeed is to be expected, the most obvious effect of isolating embryos and briefly incubating them in vitro is on their subsequent cleavage rate, which appears to be detrimentally influenced by even the minimal handling of this material. However, there appears to be no apparent long-term effect on subsequent embryonic viability in the case of controls, and even in the case of the tetraploids high rates of implantation and early postimplantation embryonic development are the norm. More advanced development, however, is restricted, presumably because of genetic problems, rather than as a consequence of the initial handling of this material. Kaufman et al. ('89), for example, recovered 94.4% normal control embryos on day 10 that had been transferred at the 1-cell stage. This is an indication of the efficiency of such a procedure.

Our results therefore show that under matched experimental conditions, tetraploid mouse embryos develop during the preimplantation period at the same rate as diploids despite their reduced cell number. This finding would seem to suggest that the hypothesis proposed by Tarkowski et al. ('77; see also Dyban and Baranov, '87), namely that the developmental retardation in the tetraploid embryos primarily results from their reduced proliferative activity, is not applicable at least in relation to their preimplantation development. Similarly, the proposal put forward by Epstein ('86) to explain the poor postimplantation development of tetraploids, namely that both the reduced rate of cell division and consequent reduced cell number in these embryos must result

in an imbalance between the physiological and morphometric requirements of the postimplantation embryo, also needs to be re-evaluated in the light of our findings.

While the size of individual cells in the tetraploids is likely to be larger than those of diploids (Snow, '75), and this may indeed interfere with critical spatial events during the postimplantation period, considerable flexibility exists in the early postimplantation period for the regulation of cell number (Tarkowski, '63; Mintz, '71; Buehr and McLaren, '74). It is possible, however, that while the system may also be sufficiently flexible to accommodate the reduced cell number of the tetraploid embryos during the early postimplantation period, secondary detrimental effects, for example, in relation to critical spatial relationships, may manifest themselves at later stages of gestation, which might lead to the death of these embryos.

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# Cleavage Rate of Haploid and Diploid Parthenogenetic Mouse Embryos During the Preimplantation Period

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**ABSTRACT** The lack of a paternal genome in parthenogenetic embryos clearly limits their postimplantation development, but apparently not their preimplantation development, since morphologically normal blastocysts can be formed. The cleavage rate of these embryos during the preimplantation period gives a better indication of the influence of their genetic constitution than blastocyst formation. Conflicting results from previous studies prompted us to use a more suitable method of following the development of haploid and diploid parthenogenetic embryos during this period.

Two classes of parthenogenetic embryos were analysed following the activation of oocytes *in vitro* with 7% ethanol: 1) single pronuclear (haploid) embryos and 2) two pronuclear (diploid) embryos. Each group was then transferred separately during the afternoon to the oviducts of recipients on the 1st day of pseudopregnancy. Control (diploid) 1-cell fertilised embryos were isolated in the morning of finding a vaginal plug, and transferred to pseudopregnant recipients at approximately the same time of the day as the parthenogenones. Embryos were isolated at various times after the HCG injection to induce ovulation, from each of the three groups studied. Total cell counts were made of each embryo, and the log mean values were plotted against time. The gradient of the lines indicated that 1) the cell doubling time of the diploid parthenogenones was  $12.25 \pm 0.34$  h, and was not significantly different from the value obtained for the control group ( $12.74 \pm 1.17$  h), and that 2) the cell doubling time of the haploid parthenogenones ( $15.25 \pm 0.99$  h) was slower than that of the diploid parthenogenones and the control diploid group. It appears from these findings that the ability of diploid parthenogenetic embryos to divide normally during the preimplantation period does not require the presence of a paternal genome. Diploid parthenogenones would, therefore, appear to be at a developmental advantage over haploid parthenogenones at the time of implantation, as their cell number is greater, and it is likely that this is related to the total number of cells that contribute to their inner cell mass.

**Key Words:** Mouse, Diploid control embryos, Paternal genome

## INTRODUCTION

Preimplantation parthenogenetic embryos provide a useful means of studying a parental influence on development, as well as the possible effect of ploidy, without the technical difficulties involved in nuclear manipulations.

Parthenogenetic embryos by definition develop without any contribution from a male, and can be produced experimentally in mammals by a variety of means (see Kaufman, 1983, for review). These embryos differ from gynogenetic embryos, in which spermatozoa act as the activating stimulus, though the male genome is subsequently eliminated and takes no further part in the development of the embryo.

While some postimplantation development has been achieved in both diploid (Kaufman et al., 1977) and haploid parthenogenetic embryos (Kaufman, 1978), it would appear that successful completion of development needs both a maternal and paternal genetic component (McGrath and Solter, 1984; Mann and Lovell-Badge, 1984; Barton et al., 1984). However, blastocyst formation can be readily achieved in diploid gynogenones and androgenones (Modliński, 1980; Borsuk, 1982; McGrath and Solter, 1984; Kaufman et al., 1989), and in haploid and diploid parthenogenones (Witkowska, 1973; Kaufman and Sachs, 1976; Kaufman, 1981). While it appears from a superficial analysis of these embryos that during the preimplantation period no obvious detrimental effect of the absence of either a maternal or paternal genome occurs, an analysis of cell number and cleavage rate may, however, provide a means of establishing whether this is indeed the case.

Haploid parthenogenones are believed to develop more slowly in tissue culture than diploid parthenogenones during the preimplantation period (Kaufman and Sachs, 1976; Kaufman, 1981). A similar situation has been reported in the case of haploid and diploid gynogenones and androgenones (Modliński, 1975, 1980; Borsuk, 1982). Digynic triploid mouse embryos are also believed to have a cell cycle time that is about 10% longer than that of normal diploid embryos (Tak-

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agi and Sasaki, 1976). Edwards (1958) observed, however, that the triploid preimplantation mouse embryos in his study cleaved at a similar rate to that of normal diploid embryos. By contrast, preimplantation tetraploid mouse embryos develop at the same rate as diploids, while possessing approximately half the cell number of diploid embryos at each stage of development (Smith and McLaren, 1977; Eglitis and Wiley, 1981; Henery and Kaufman, 1991). Octaploid mouse embryos also develop at the same rate as diploids (Winkel and Nuccitelli, 1989).

Since development *in vivo* is generally regarded as being preferable in most regards to development *in vitro* (Bowman and McLaren, 1970; Streffer et al., 1980; Harlow and Quinn, 1982), we decided to undertake a detailed evaluation of the cleavage rate *in vivo* of preimplantation haploid and diploid parthenogenones following their transfer to appropriate recipients. Witkowska (1973) activated newly ovulated mouse eggs *in situ* with an electric shock, and examined the development of the parthenogenetically activated eggs on subsequent days of gestation. However, this study was difficult to interpret, because a mixture of haploid and diploid parthenogenetic embryos almost invariably resulted from this activation technique (Tarkowski et al., 1970). We believe that it is preferable to use *in vitro* activation techniques, as this allows the selective transfer of recently activated haploid or diploid parthenogenetic embryos to pseudopregnant recipients, and allows their individual development to be analysed and compared with that of other classes of parthenogenones, as well as with control diploid embryos studied under similar experimental conditions.

## MATERIALS AND METHODS

Eight- to 12-week-old (C57BL  $\times$  CBA) F1 hybrid female mice were superovulated with 5 i.u. PMSG followed 48 hours later by 5 i.u. HCG. Females were autopsied at 19–19.5 hours after the HCG injection. The oviducts were then removed and the individual cumulus masses containing the ovulated oocytes released into a watchglass containing M2 tissue culture medium (Quinn et al., 1982). The cumulus masses were transferred via a Pasteur pipette to a watchglass containing about 1 ml of a freshly prepared 7% solution of Analar quality ethanol in phosphate buffered saline (PBS) and retained in this solution for 7 minutes at 37°C (Kaufman, 1982). The cumulus masses were then washed through two changes of M2 and one of M16 tissue culture medium (Whittingham, 1971) before being transferred to drops of M16 under paraffin oil and incubated for 4–5 hours at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. At the end of this time, the adherent cumulus cells were removed with hyaluronidase (Kaufman, 1978), and the various classes of parthenogenone induced separated into different groups.

Four classes of parthenogenone could be determined at this stage, namely (a) oocytes which contained a single (haploid) pronucleus, having previously ex-

truded a second polar body, (b) oocytes which contained two (haploid) pronuclei in the absence of second polar body extrusion, (c) oocytes which underwent "immediate cleavage" in which two equal-sized blastomeres had formed, each containing a single (haploid) pronucleus, one of the blastomeres representing the second polar body, and (d) oocytes in which a single (diploid) pronucleus developed in the absence of second polar body extrusion. We were concerned in this study with the preimplantation development of groups a and b.

Between 10–12 embryos belonging to either group a or b were then transferred unilaterally to the oviducts of recipients on the afternoon of the 1st day of pseudopregnancy (i.e., on the day of finding a vaginal plug after spontaneously cycling females had been mated to proven sterile vasectomised males). The recipients were anaesthetised with tribromoethanol (Avertin, Winthrop, dose 0.02 ml/g body weight of a freshly prepared 1.2% solution of Avertin dissolved in 0.9% saline).

In the control series, F1 hybrid female mice were superovulated and mated individually with fertile homozygous Rb(1.3)1Bnr males. The presence of a vaginal plug the next morning was taken as evidence of mating, and considered to be the 1st day of gestation. These females were autopsied on the morning of finding a vaginal plug and the oviducts were removed. The individual cumulus masses containing the fertilized eggs were released into PBS and then transferred to a plastic tissue culture dish containing drops of M16 with hyaluronidase (0.001 g/ml). The individual eggs were then washed four times in M16 under paraffin oil and retained for 3–4 h in an incubator maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> in air.

These 1-cell fertilised (control) embryos were then transferred unilaterally to the oviducts of recipients in the afternoon on the 1st day of pseudopregnancy, as described above. At specific time points after the transfers were carried out, the recipient mice, which contained haploid or diploid parthenogenetic or control diploid embryos, were killed by cervical dislocation. The oviducts or uteri on the operated side were removed and flushed through with PBS containing 4% bovine serum albumin (BSA) in order to recover the transferred embryos. In all instances, note was taken of the gross morphological appearance of individual embryos, and from which location within the reproductive tract they were recovered. Preparations were made of these embryos using the air-drying technique described by Tarkowski (1966), and the spreads were then stained with 10% giemsa to enable accurate cell counts to be made.

## RESULTS

The findings from this study are shown in Table 1 and Figures 1 and 2. The data can be considered first in relation to total cell count for the three groups and second in relation to the location and morphological appearance of the embryos.

TABLE 1. Mean Cell Doubling Time for (i) Control Fertilized Diploid Embryos, (ii) Diploid Parthenogenetic Embryos, (iii) Haploid Parthenogenetic Embryos

| Group                            | Time of isolation<br>(hours post HCG) | No. of embryos<br>transferred | No. recovered<br>(healthy) | No. analysed to<br>establish<br>cell number | Mean cell<br>number<br>± SEM |
|----------------------------------|---------------------------------------|-------------------------------|----------------------------|---|------------------------------|
| (i) Control diploids             | 48                                    | 58                            | 61 <sup>a</sup>            | 58  | 2.22 (± 0.08)                |
|                                  | 75                                    | 55                            | 51                         | 46  | 6.46 (± 0.51)                |
|                                  | 80                                    | 70                            | 56                         | 50  | 13.74 (± 0.67)               |
|                                  | 96                                    | 65                            | 53                         | 41  | 30.51 (± 2.44)               |
|                                  | 105                                   | 106                           | 57                         | 41  | 51.90 (± 3.32)               |
| (ii) Diploid<br>parthenogenones  | 50                                    | 46                            | 46                         | 46  | 2.30 (± 0.11)                |
|                                  | 76                                    | 69                            | 49                         | 48  | 9.79 (± 0.52)                |
|                                  | 92                                    | 76                            | 46                         | 41  | 27.00 (± 1.54)               |
|                                  | 99                                    | 130                           | 73                         | 61  | 35.20 (± 1.99)               |
| (iii) Haploid<br>parthenogenones | 50                                    | 60                            | 51                         | 50  | 2.10 (± 0.06)                |
|                                  | 76                                    | 123                           | 82                         | 64  | 8.53 (± 0.38)                |
|                                  | 93                                    | 91                            | 64                         | 55  | 16.58 (± 0.79)               |
|                                  | 99                                    | 128                           | 91                         | 73  | 16.62 (± 1.07)               |
|                                  | 104                                   | 53                            | 32                         | 21  | 26.09 (± 4.14)               |

<sup>a</sup>It is assumed that 3 of these embryos must have resulted from the parthenogenetic activation of the recipients' eggs, due to the activating stimulus of the anaesthetic employed during the transfer procedure (Kaufman, 1975; Kaufman and Sachs, 1975).

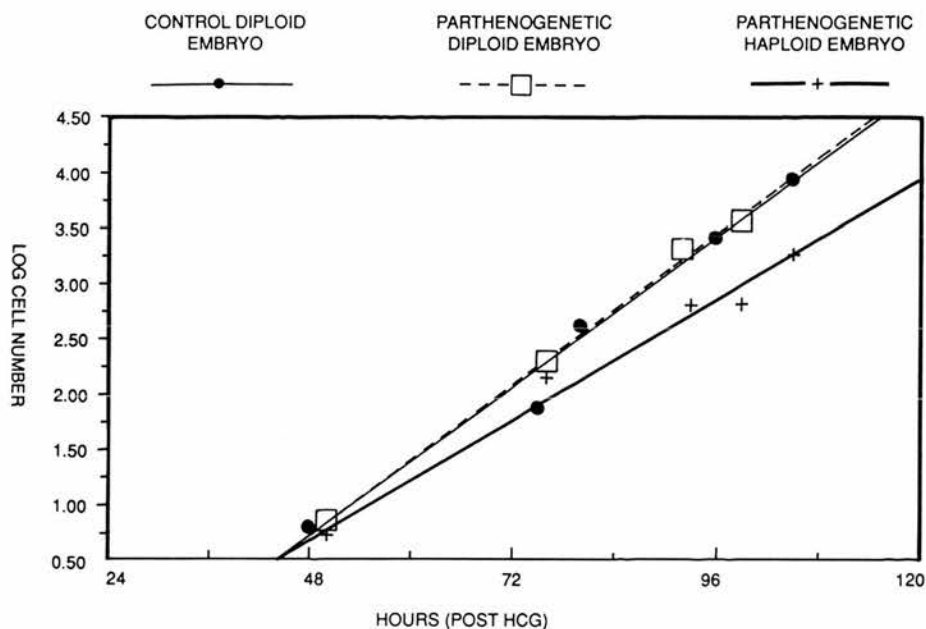


Fig. 1. Log total cell number of embryos isolated at various times during the preimplantation period, plotted against time after HCG injection to induce ovulation in (i) control diploid embryos, (ii) diploid parthenogenetic embryos, and (iii) haploid parthenogenetic embryos. —●— = control diploid embryo; ---□--- = diploid parthenogenetic embryo; —+— = haploid parthenogenetic embryo.

#### Cell Doubling Time for Fertilised Diploid Control Embryos and In Vitro Activated Two-Pronuclear Diploid and Single-Pronuclear Haploid Parthenogenetic Embryos After Isolation at the 1-Cell Stage and Subsequent Transfer to Pseudopregnant Recipients

Directly after the embryos from these groups had been isolated from the pseudopregnant recipients at specific times after the HCG injection for inducing

their ovulation, their cell number was established. The values obtained were converted to natural logarithms, and were plotted graphically against time after HCG injection (see Fig. 1). Regression lines were drawn for each class of embryo, and this enabled their individual cell doubling time to be calculated. For the diploid control group, the cell doubling time between 48 and 105 h after the HCG injection was found to be  $12.74 \pm 1.17$  h.

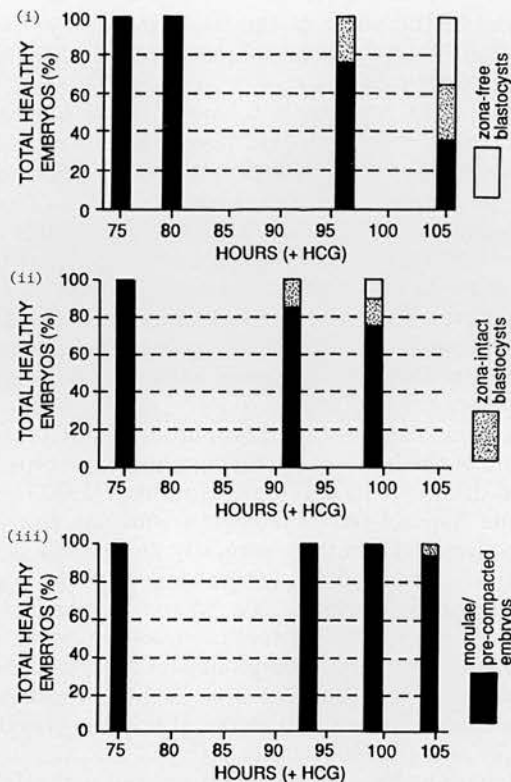


Fig. 2. Morphological appearance of embryos isolated at various times during the preimplantation period, plotted against time after HCG injection to induce ovulation. (i): Control diploid embryos; (ii): Diploid parthenogenetic embryos; (iii): Haploid parthenogenetic embryos.

The cell doubling time for the two-pronuclear diploid parthenogenetic group between 50 and 99 h after the HCG injection was found to be  $12.25 \pm 0.34$  h. This was not statistically different from the value obtained for the control series (i) ( $P > .05$ ).

The cell doubling time for the single-pronuclear haploid parthenogenetic group between 50 and 104 h after the HCG injection was  $15.25 \pm 0.99$  h. The gradient of this line was statistically different from the gradient of the diploid parthenogenetic embryos ( $P < .05$ ).

#### The Location and Morphological Appearance of Control and Parthenogenetic Embryos at Various Times After Their Transfer to Pseudopregnant Recipients

**i. Fertilised diploid control embryos.** All of the healthy embryos recovered from the diploid control group at 80 h after the original HCG injection were compacted morulae or in a few cases precompacted embryos with a mean cell number ( $\pm$ S.E.M.) of  $13.74 \pm 0.67$ . They were exclusively located in the oviduct. By 96 h, nearly 23% of all the healthy embryos were blastocysts, and the rest were morulae or in a few cases precompacted embryos. Embryos could be found in both the oviduct and the uterus at this time. By 105 h, all the embryos were located in the uterus and nearly

37% of these were zona-free blastocysts, 29% were expanded zona-intact blastocysts, and the remaining embryos (34%) were morulae or (rarely) precompacted embryos.

**ii. Diploid parthenogenetic embryos.** The diploid parthenogenones followed a similar progress to i above. At 76 h after the original HCG injection, all the healthy embryos recovered were morulae (mean cell number  $\pm$  S.E.M.  $9.79 \pm 0.52$ ), and were all located in the oviduct. By 92 h, morulae and the first blastocysts were observed, and they constituted 15% of all the healthy embryos recovered. Embryos could be found at this time in both the oviduct and the uterus. By 99 h, zona-free blastocysts were recovered from the uterus, although the majority of embryos were zona-intact blastocysts and morulae, and were recovered from both the oviduct and the uterus.

**iii. Haploid parthenogenetic embryos.** In the case of the haploid parthenogenones, in the period of time studied, embryos had progressed only to the morula stage (and rarely to the blastocyst stage, approximately 5%) or were precompacted embryos. Curiously, by 76 h after the original HCG injection, embryos were recovered from both the oviduct and the uterus, and had progressed further in the female tract than the embryos of groups i and ii, at approximately the same time after the HCG injection.

The morphological appearance, whether precompacted embryos and/or morulae, zona-intact or zona-free blastocysts, of embryos from groups i–iii, above, at various times after the HCG injection to induce ovulation, is illustrated in Figure 2.

#### DISCUSSION

We have found that the parthenogenetically activated single-pronuclear (uniform) haploid mouse embryos had a slower cleavage rate during the preimplantation period than that of heterozygous diploid parthenogenetic embryos and fertilised control diploid embryos when all three groups were transferred to pseudopregnant recipients. The diploid parthenogenones, however, appeared to cleave at approximately the same rate as the fertilised control diploid embryos. This result suggests that while the lack of one genetic component (in the case of the haploid parthenogenones) increases the duration of the cell cycle and consequently slows down their development, the presence of two maternally derived genetic components does not appear to do so. Indeed, at least during the preimplantation period, the presence of such an abnormal genome appears to have little obvious detrimental effect in terms of cleavage rate and the ability of these embryos to form apparently morphologically normal blastocysts. An alternative explanation that may partly account for the slower cleavage rate of the single-pronuclear haploid compared to the diploid parthenogenones and the diploid control embryos relates to the sub-optimal nuclear-cytoplasmic ratio of the haploid embryos. This is consistent with the findings of McGrath and Solter (1986) who compared, in an elegant series of experi-



ments, the developmental ability of single-pronuclear haploid embryos produced by the removal of a single male or female nucleus with haploid embryos that underwent normalization of their nuclear-cytoplasmic ratio by removing a single pronucleus and additionally half the cytoplasmic volume of the zygote. They observed an increased proportion of the cytoplasm-depleted haploid embryos developed to the morula/blastocyst stage. This finding was similar to the earlier observation that an increased proportion of parthenogenones that underwent either immediate cleavage or delayed cleavage (and thus normalized their nuclear-cytoplasmic ratio) developed beyond the 4-cell stage when compared to single-pronuclear haploid embryos that possessed a decreased nuclear-cytoplasmic ratio (Kaufman and Sachs, 1976; Kaufman, 1981). Similarly, others have observed that a greater proportion of haploid embryos produced by zygote bisection (Tarkowski and Rossant, 1976; Tarkowski, 1977) complete preimplantation development than haploids produced by pronuclear removal alone (Modliński, 1975, 1980).

However, Kaufman and Sachs (1976) noted that under their culture conditions, unlike the finding in this study, fertilised control diploid embryos cleaved slightly more rapidly than did their diploid parthenogenones. Since, as has been indicated previously, *in vitro* development is generally considered suboptimal when compared with *in vivo* development (Bowman and McLaren, 1970), it is possible that those embryos which may already be at a genetic disadvantage may be more sensitive to the environmental conditions *in vitro* than genetically normal embryos.

Haploid gynogenetic and androgenetic mouse embryos produced by microsurgically removing one pronucleus from a fertilised embryo showed a markedly delayed rate of development during the preimplantation period when compared with that of "homozygous" diploid embryos produced by the diploidisation of similar eggs following their exposure to cytochalasin B (Modliński, 1975, 1980; Borsuk, 1982). Most of the haploid embryos in these studies remained at the morula stage and failed to progress to the blastocyst stage, whereas the homozygous diploid embryos regularly formed blastocysts (Borsuk, 1982). It should be noted, though, that only the haploid eggs in which the male pronucleus was removed (i.e., the gynogenones) survived to the 5th day in culture. One possible explanation was proposed for this finding, namely that damage was more likely to have been sustained by the cytoskeletal elements and vitelline membrane during the removal of the female (but not the male) pronucleus, and that this may have had a detrimental effect on the viability of the androgenones (Modliński, 1975). In addition, since about 25% of diploid androgenetic preimplantation embryos possess a YY sex chromosome constitution, they would not in any case be expected to reach the blastocyst stage (Solter, 1988).

The haploid embryos analysed by Edwards (1958) apparently had fewer cells than were present in control diploid embryos. This finding, however, was attributed

to a delay in the onset of the first cleavage division, rather than being a consequence of their having a slower cleavage rate.

The fact that less haploid than diploid parthenogenones reach the blastocyst stage (Kaufman, 1981, and this study) and that their cell number even when they do so is much reduced in comparison to diploid parthenogenones when maintained under similar conditions either *in vivo* or *in vitro*, may mean that they are less capable of evoking a decidual reaction and implanting in the uterus during the limited period of time that the uterus is in a receptive phase. In this study, while most of the control diploid embryos and the diploid parthenogenetic embryos were at the blastocyst stage at approximately 99 hours after the HCG injection, none of the haploid parthenogenetic embryos had achieved this stage by this time. Kaufman (1981) noted that some haploid parthenogenetic morulae that initially appeared to compact normally go through a "decompaction" phase before progressing to form apparently normal blastocysts. This "decompaction" phase appears to be a feature of some haploid embryos, and does not appear to occur in diploid parthenogenetic embryos nor in normal fertilised diploid embryos. Haploid embryos clearly have a reduced total number of cells at this time, and this inevitably reduces the number of cells likely to be located in their inner cell mass (ICM). By the time that implantation would normally occur, the total number of ICM cells present may be insufficient to allow the development of the embryo proper to occur, despite the presence of a relatively normal decidual response (Ansell and Snow, 1975; Snow et al., 1976). Haploid parthenogenones with few or no ICM cells have been observed previously, although it is believed that diploid parthenogenones almost always contain ICM cells (Kaufman, 1978).

We observed in this study that heterozygous diploid parthenogenones cleaved at the same rate as control diploid embryos (see Fig. 1). In earlier studies, it has been reported that their potential for postimplantation development is restricted, and that progress beyond the early forelimb bud stage has not so far been observed (Kaufman et al., 1977). This finding is believed to result from the absence of a paternal genome in these embryos, since both sets of parental genomes are believed to be essential for successful development. Our study would appear to indicate that the presence of two maternally derived genomes, at least during the preimplantation period, has no obvious detrimental effect on the cleavage rate of diploid parthenogenones.

Our results show that mouse parthenogenetic heterozygous diploid embryos cleave at the same rate as control diploid embryos when maintained under similar conditions *in vivo*, while haploid parthenogenetic embryos cleaved more slowly. It is of interest that the duration of the first cleavage mitosis is almost identical in diploid parthenogenetic and fertilised embryos, while the duration of this mitosis in haploid parthenogenones is significantly slower (Kaufman, 1983). If a similar pattern also occurs in subsequent divisions, this

might provide a partial explanation for the present findings, although it should be noted that in that study both the haploid and diploid parthenogenones entered the first cleavage mitosis at approximately the same time after activation (Kaufman, 1983).

Since we, and others, have found that mouse preimplantation homozygous tetraploid embryos produced by blastomere fusion at the 2-cell stage (these embryos are genetically balanced) cleave at the same rate as diploid controls (Smith and McLaren, 1977; Eglitis and Wiley, 1981; Henery and Kaufman, 1991), it will be of considerable interest to analyse the cleavage rate of triploid embryos (which are inevitably genetically unbalanced) to investigate whether any relationship exists between their ploidy and cell number at different times during the preimplantation period, as has been suggested by various authors (e.g., Edwards, 1958). It will in addition be of particular interest to establish whether diandric and digynic triploid embryos have a similar cleavage rate when these embryos are retained under identical conditions either *in vivo* or *in vitro*.

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# Cleavage Rate of Diandric Triploid Mouse Embryos During the Preimplantation Period

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**ABSTRACT** The postimplantation development of human and animal triploid embryos is well documented, but there is little informative data on their preimplantation development. An analysis of cell number at appropriate times during this period and thus their cleavage rate would give an indication of the potential triploids have for further development and may explain some problems associated with their postimplantation development. To rule out any effects of technical procedures on cleavage rate, appropriate controls were used. Diandric triploid embryos were produced using standard micromanipulatory techniques, which involved the injection of a male pronucleus into a recipient one-cell-stage embryo. The karyoplast was fused to the cytoplasm by electrofusion, and the resulting trippronucleate diandric triploid embryos were transferred to appropriate pseudopregnant recipients. At specific times after the transfer, the embryos were recovered and cell numbers established. The results were plotted and regression lines drawn. Three controls were used 1) micromanipulated diploid embryos from which the male pronucleus had been removed and immediately reinserted and fused to restore diploidy, 2) diploid embryos that had been briefly incubated in cytochalasin D and colcemid to find out the effects these agents had on development, and 3) diploid embryos that had been isolated and briefly incubated in tissue culture medium. All embryos were subsequently transferred to recipients. After isolation at specific times during the preimplantation period, cell numbers were also established and the results plotted. The cell doubling time of the diandric triploid embryos was 13.55 hr ( $\pm 1.25$ ), and this was not significantly different from the various controls. The cell doubling time of 1) the micromanipulated controls was 12.12 hr ( $\pm 1.16$ ), 2) the control embryos incubated in cytoskeletal inhibitors 10.87 hr ( $\pm 0.75$ ), and 3) the group that was briefly incubated in tissue culture 12.43 hr ( $\pm 0.74$ ). There was no significant effect of manipulation or incubation in cytoskeletal inhibitors on cleavage rate. Our findings indicate that triploid embryos divide at the same rate as diploid embryos during the preimplantation period. © 1992 Wiley-Liss, Inc.

**Key Words:** Diploid embryos, Trippronucleate embryos, Cell doubling time

postimplantation development has been reasonably well documented in experimental animals (Fischberg and Beatty, 1952; Vickers, 1969; Wróblewska, 1971; Takagi and Oshimura 1973; Takagi and Sasaki, 1976; Baranov, 1976; Speirs and Kaufman, 1989a,b; Kaufman et al., 1989a; and, for recent reviews of triploidy in mice, see Dyban and Baranov, 1987; Kaufman, 1991) and in humans (Carr, 1971a,b; Niebuhr, 1974; Beatty, 1978; Jacobs et al., 1982).

Digynic triploidy has been particularly well analysed because it is technically easier to produce experimentally than diandric triploidy (Niemierko and Opas, 1978). In particular, both cytochalasin B and more recently cytochalasin D, which are cytokinesis inhibitors, have been useful in the production of digynic triploid mouse embryos since exposure of recently fertilised embryos to one or the other of these agents can inhibit the extrusion of the second polar body (Niemierko, 1975, 1981; Surani and Barton, 1983; Speirs and Kaufman, 1989a,b). The postimplantation development of experimentally induced and spontaneously occurring digynic triploid mouse embryos is limited, and most die between the eighth and tenth days of gestation. Furthermore, they commonly display various abnormalities of the neural tube and heart as well as a reduction in size when compared to fertilised diploid embryos of a similar developmental stage (see above for references). In many strains, however, the triploidy syndrome is seen, in which only an empty gestational sac is formed (Wróblewska, 1971).

Diandric triploidy can occur, though rarely, through the fertilisation of a normal egg by a diploid spermatozoon, or more commonly through dispermy, due to the fertilisation of an egg by two haploid spermatozoa. Approximately 85% of human triploids are believed to be of diandric origin and only 15% of digynic origin (Jacobs et al., 1982; Surti, 1987), although the proportionate incidence of these two classes varies considerably between species (Dyban and Baranov, 1987). Recently, in our laboratory, diandric triploid mouse embryos have been routinely produced by nuclear manipulation (Kaufman et al., 1989a,b). Successful postimplantation development has been achieved, but only to about 10

## INTRODUCTION

There is little informative data on the preimplantation development of triploid embryos, yet their early

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days of gestation. The diandric triploid embryos in these studies generally appeared to be morphologically normal although smaller than fertilised embryos at similar developmental stages (Kaufman et al., 1989a).

The reduction in size of postimplantation triploid embryos compared with developmentally matched controls may result from their reduced cell number, possibly due to their slower cleavage rate during the preimplantation period. Beatty and Fischberg (1951) and Edwards (1958) both found a reduction in cell number in triploid mouse embryos at 3.5 days of gestation compared with diploid embryos. Takagi and Sasaki (1976) proposed that triploids had a longer cell cycle time than diploid embryos. These authors estimated that the cell cycle time in the digynic triploid embryos in their study was about 10% longer than that of their controls; they hypothesised that, by the tenth cleavage division, the cell number in triploids would become one-half that of diploids and one-fourth after 20 cleavage divisions. Niemierko (1975), by contrast, believed that any reduction in cell number in the triploids in her studies probably resulted from the stress of the treatment procedure rather than as a result of triploidy per se. Human triploid cells maintained in tissue culture are also reported to have had a normal cleavage rate (Kuliev et al., 1975; Hassold and Sandison, 1983).

Since diandric triploid mouse embryos produced experimentally by nuclear manipulation survive and are usually morphologically normal until the limb-bud stage, we decided to investigate whether their cleavage rate during the preimplantation period was similar to that of controls when both groups were exposed to identical experimental conditions initially *in vitro* and subsequently *in vivo* following their transfer to appropriate recipients. A reduction in cell number during this period may account for the smaller size of the embryos later in gestation.

## MATERIALS AND METHODS

To satisfy ourselves that the preimplantation diandric triploid embryos we were analysing were capable of normal early postimplantation development, an initial series of experiments was undertaken. To show unequivocally that our procedure for producing diandric triploid embryos was satisfactory, Rb(1.3)1Bnr male mice were used. The genotype of homozygous Rb(1.3)1Bnr males ( $2n = 38$ ) contains two large metacentric chromosomes, being Robertsonian translocations involving chromosomes 1 and 3 (cf. in normal mice  $2n = 40$ ). Consequently, following fertilisation by spermatozoa from these males, the paternally derived haploid genome contains 18 acrocentric and one large metacentric "marker" chromosome. Thus diandric triploid embryos, which have a normal female chromosome complement associated with two paternally derived haploid chromosome sets derived from the homozygous Rb(1.3)1Bnr males, would invariably be expected to contain a total complement of 58 chromosomes, two of which would be the large metacentric "marker" chromosomes.

Eight- to twelve-week-old (C57BL  $\times$  CBA) $F_1$  hybrid female mice were injected with 5 i.u. pregnant mare serum gonadotrophin (PMSG) followed 48 hr later by 5 i.u. human chorionic gonadotrophin (HCG). Shortly after the HCG injection, the females were caged with fertile homozygous Rb(1.3)1Bnr males. Early the following morning, the females were checked for the presence of a vaginal plug, and this was taken as evidence of mating. The morning of finding a vaginal plug was considered the first day of pregnancy.

Early pronucleate-stage, fertilised eggs were isolated at about 10 AM on the day of finding a vaginal plug. In these early fertilised eggs, the female pronucleus is always located in close proximity to the second polar body, while the male pronucleus is located elsewhere in the cytoplasm, but usually in the subcortical zone located at the periphery of the egg.

The fertilised eggs were incubated for 45 min in M2 medium (Quinn et al., 1982) supplemented with 1  $\mu$ g/ml cytochalasin D and 1  $\mu$ g/ml colcemid prior to microinjection. Male pronuclei were isolated with a small volume of cytoplasm from "donor" eggs and inserted into the perivitelline space of "recipient" one-cell-stage, fertilised eggs using standard micromanipulatory techniques (McGrath and Solter, 1983). Batches of these fertilised eggs with injected donor pronuclei were washed through drops of M16 medium (Whittingham, 1971) and then incubated for 1 hr in M16 at 37°C in an atmosphere of 5% CO<sub>2</sub> in air.

The injected pronuclei were then fused to the fertilised egg cytoplasm by electrofusion. The embryos to be fused were transferred into a nonelectrolyte solution consisting of 0.3 M mannitol (Kubiak and Tarkowski, 1985), and a similar solution was also present in the fusion chamber. The latter consisted of a plastic tissue culture dish that had two platinum wires each of 250  $\mu$ m diameter fixed approximately parallel to each other on the bottom of the fusion dish, with a space of about 600  $\mu$ m between them. The ends of the platinum wires were connected to a digitimer pulse stimulator set at 100 mV, with a pulse duration of 50  $\mu$ sec. The manipulated embryos were individually placed between the wires to ensure that the injected pronucleus was orientated approximately parallel to the wires, and the pulse stimulator was triggered. The embryos were removed immediately and washed through four drops of M16 and then returned to the incubator.

Fusion usually occurred within 1 hr. These diandric triploid trippronucleate embryos were then transferred unilaterally to the oviducts of recipients on the first day of pseudopregnancy (i.e., on the first day of finding a vaginal plug after spontaneously cycling females had been mated to proven sterile vasectomised males). The recipients were anaesthetised with tribromoethanol (Avertin; Winthrop; dose 0.02 ml/g body weight of a freshly prepared 1.2% solution of Avertin dissolved in 0.9% saline).

These recipients were then autopsied at about mid-day on days 9–12 of gestation, and the numbers of implantation sites present, resorptions, and embryos re-

covered were noted. Cytogenetic analyses of the extraembryonic membranes—principally the yolk sac—of the intact conceptuses were made using a modification of the technique described by Evans et al. (1972), in order to confirm that they were indeed diandric triploid embryos.

### Experimental Series: Preimplantation Development of Diandric Triploids

Another group of superovulated  $F_1$  hybrid females was mated to fertile homozygous Rb(1.3)1Bnr males, and the male pronuclei were inserted into the perivitelline space of recipient eggs as described above. After electrofusion, these diandric triploid trippronucleate embryos were also transferred unilaterally to the oviducts of recipients. At various times between 49 and 100 hr after the HCG injection, the recipients were killed by cervical dislocation. The oviducts or uteri on the operated side were removed and flushed through with phosphate-buffered saline (PBS) containing 4% bovine serum albumin (BSA) to recover the transferred embryos.

In preliminary experiments in this series, the embryos were incubated for 3–4 hr in the presence of colcemid to determine their chromosome constitution. This procedure was not particularly satisfactory, although the triploid status of the embryos could usually be determined. The relatively poor quality of many of the preparations, however, often made it difficult to recognise both “marker” chromosomes in every spread. It was necessary therefore in this series to assume that the efficiency of our microinjection technique was extremely high, as observed from the findings in our postimplantation “control” study.

In all instances, note was taken of the gross morphological appearance of individual embryos; whether they were at cleavage stages, precompacted, or compacted morulae or blastocysts; and from which location within the reproductive tract they were recovered. Preparations were made of these embryos using the air-drying technique described by Tarkowski (1966) and the spreads were then stained with 10% giemsa to allow for accurate cell counts. In each of the control series,  $F_1$  hybrid females were mated to homozygous Rb(1.3)1Bnr males.

#### Control Series I

In this series, the male pronucleus was removed via the injection pipette and then reinserted into the perivitelline space, where, after electrofusion, it subsequently fused to the cytoplasm to restore the diploid status of these embryos. The embryos were then transferred to pseudopregnant recipients and isolated at specific time points after the transfer, when their total cell number was established as described previously.

#### Control Series II

In this series, we investigated whether incubation of early embryos in cytochalasin D and colcemid had any detrimental effect on cleavage rate, because exposure

to these agents invariably occurs during nuclear manipulation. Pronuclear eggs were isolated and incubated in drops of M2 supplemented with 1  $\mu$ g/ml cytochalasin D and 1  $\mu$ g/ml colcemid for 1.5–2 hr. They were then washed four times in M16 under paraffin oil and retained for a further 1 hr in an incubator maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. They were then transferred unilaterally to recipients and isolated at specific time points later, when the appearance and location of the embryos recovered was noted. Total cell counts were then made as described above.

#### Control Series III

This series was a control for the surgical procedure of transferring embryos into pseudopregnant recipients. One-cell, pronucleate-stage, fertilised embryos were recovered on the morning of finding a vaginal plug and retained in M16 in an incubator for 3–4 hr. They were then transferred unilaterally in batches of approximately 10 to the oviducts of recipients, and at specific times later they were isolated and their total cell counts established.

## RESULTS

### Postimplantation Development of Diandric Triploid Mouse Embryos

The findings in relation to the postimplantation development of diandric triploid mouse embryos in this study are presented in Table 1. The most advanced embryos were recovered on day 11.5 of gestation, and of these 10 were partially “turned” or “turned” early forelimb bud stage embryos, each of which possessed approximately 25–30 pairs of somites. No morphologically normal embryos were recovered after this time; only embryonic and extraembryonic remnants were seen. Diandric triploidy was confirmed cytogenetically in 89.5% of all of the embryos recovered.

#### Preimplantation Development

There are four groups in this study, namely, a diandric triploid group and three different controls. Cell doubling times are given for each group, and a comparison is also made between groups to rule out the possible effects of specific technical factors on their cleavage rates. The location and morphological appearance of these embryos at the various time points studied are also described.

#### Cell Doubling Times

After the embryos from each group had been isolated from the recipients at specific times after the original HCG injection, their total cell count was established. The values obtained were converted into natural logarithms and plotted against time after HCG. Regression lines were drawn, and this allowed a cell doubling time for each group to be established.

The diandric triploid embryos had a cell doubling time of 13.55 hr ( $\pm 1.25$ ) between 49 and 100 hr after the original HCG injection. For the micromanipulated dip-

TABLE 1. Postimplantation Development of Diandric Triploid Embryos Following Their Transfer to Recipients

| Day of gestation at autopsy | No. recipients | No. embryos transferred | No. implants | No. resorptions | No. embryos | Diandric triploidy confirmed <sup>a</sup> | Partially "turned"/ "turned" embryos |
|-----------------------------|----------------|-------------------------|--------------|-----------------|-------------|---|--------------------------------------|
| 8.5                         | 3              | 27                      | 18           | 10              | 8           | 6   | 0                                    |
| 9.5                         | 4              | 35                      | 32           | 19              | 13          | 12  | 2                                    |
| 10.5                        | 3              | 19                      | 18           | 11              | 7           | 6   | 3                                    |
| 11.5                        | 10             | 98                      | 62           | 42              | 20          | 19  | 10                                   |

<sup>a</sup>One additional embryo was a digynic triploid, another was a diploid, and we were unable to analyse three further embryos.

loids (control series I) this was 12.12 hr ( $\pm 1.16$ ) between 55 and 102 hr after HCG. Embryos incubated briefly in cytochalasin D and colcemid (control series II) had a cell doubling time of 10.87 hr ( $\pm 0.75$ ) between 49 and 101 hr, and for the embryos isolated and incubated only in M16 (control series III) this was 12.43 hr ( $\pm 0.74$ ) between 48 and 105 hr after HCG.

The regression lines plotted for these embryos showed that diandric triploid mouse embryos produced by the technique of nuclear manipulation divide at the same rate as the micromanipulated controls. What is also evident is that the stresses of nuclear manipulation on an embryo do not appear to affect its cleavage rate detrimentally (comparison of control series I and III). The lines of regression plotted for these two groups were not significantly different from each other ( $P > 0.05$ ). A comparison of embryos that had briefly been exposed to cytochalasin D and colcemid with embryos that had not (control series II and III) was similarly not significantly different ( $P > 0.05$ ). The findings from this study are summarised in Table 2 and the regression lines presented in Figure 1.

#### Location and Morphology of Preimplantation Embryos

The diandric triploids at 72 hr after the HCG injection were found exclusively in the oviduct and were all either precompacted or compacted morulae. By 96 hr, some had reached the blastocyst stage, having a mean cell number of 17.9 ( $\pm 1.2$ ), and were located in both the oviduct and uterus. By 100 hr, 74% were blastocysts and mostly were located in the uterus. A proportion of the blastocysts were zona-free.

In the various control groups, the micromanipulated diploid controls (series I) showed a progress similar to that of the triploids, but by 102 hr after HCG only 41% were blastocysts. Nearly 50% of the embryos exposed briefly to cytochalasin D and colcemid (control series II) were blastocysts after 96 hr and by 101 hr after HCG approximately 71% had reached that stage. In the additional control group (control series III), 23% were blastocysts by 96 hr and were located in both the oviduct and the uterus. By 105 hr, all the embryos were located in the uterus, and nearly 66% of these were blastocysts. The findings from this component of the study are presented in diagrammatic form in Figure 2.

#### DISCUSSION

Our results clearly demonstrate that diandric triploid mouse embryos cleave at the same rate as control diploid embryos during the preimplantation period. An additional set of male chromosomes has no obvious detrimental effect on cleavage rate during the preimplantation period, while the absence of a set of chromosomes clearly does (Modliński, 1975, 1980; Kaufman and Sachs, 1976; Kaufman, 1981; Henery and Kaufman, 1992).

From an analysis of the morphological appearance of diandric triploid embryos in this study, it appears likely that they were healthy at least until day 11 of



TABLE 2. Preimplantation Development of Diandric Triploid and Control Mouse Embryos

| Group   | Time of isolation<br>(hr post-HCG) | No. embryos<br>transferred | No. embryos<br>recovered<br>(healthy) | No. analysed<br>to establish<br>cell number | Mean cell number<br>(SEM) |
|---|------------------------------------|----------------------------|---------------------------------------|---|---------------------------|
| 1. Diandric triploid  | 49                                 | 21                         | 21                                    | 21  | 2.00 (0.00)               |
|   | 72                                 | 57                         | 51                                    | 45  | 5.73 (0.29)               |
|   | 80                                 | 60                         | 44                                    | 41  | 9.46 (0.65)               |
|   | 96                                 | 78                         | 56                                    | 48  | 17.92 (1.16)              |
|   | 100                                | 63                         | 45                                    | 35  | 30.77 (2.21)              |
| 2. Micromanipulated diploid controls                                      | 55                                 | 20                         | 21 <sup>a</sup>                       | 19  | 2.26 (0.15)               |
|   | 72                                 | 34                         | 37 <sup>a</sup>                       | 31  | 5.55 (0.33)               |
|   | 79                                 | 44                         | 41                                    | 30  | 9.60 (0.77)               |
|   | 96                                 | 60                         | 39                                    | 34  | 22.62 (1.65)              |
|   | 102                                | 53                         | 39                                    | 31  | 33.30 (3.04)              |
| 3. Cytochalasin D/colcemid controls                                       | 49                                 | 31                         | 25                                    | 25  | 2.00 (0.00)               |
|   | 74                                 | 51                         | 46                                    | 40  | 7.40 (0.44)               |
|   | 80                                 | 47                         | 49 <sup>a</sup>                       | 44  | 12.79 (0.47)              |
|   | 96                                 | 41                         | 46 <sup>a</sup>                       | 41  | 31.34 (1.55)              |
|   | 101                                | 64                         | 51                                    | 48  | 55.46 (2.84)              |
| 4. Transferred one-cell controls (not exposed to cytoskeletal inhibitors) | 48                                 | 58                         | 61 <sup>a</sup>                       | 58  | 2.22 (0.08)               |
|   | 75                                 | 55                         | 51                                    | 46  | 6.46 (0.51)               |
|   | 80                                 | 70                         | 56                                    | 50  | 13.74 (0.67)              |
|   | 96                                 | 65                         | 53                                    | 41  | 30.51 (2.44)              |
|   | 105                                | 106                        | 57                                    | 41  | 51.90 (3.32)              |

<sup>a</sup>The additional embryos recovered above those transferred to recipients are likely to have resulted from the parthenogenetic activation of the recipients' eggs, due to the activating stimulus of the anaesthetic employed during the transfer procedure (Kaufman, 1975; Kaufman and Sachs, 1975).

gestation. The diandric triploid embryos were, however, consistently smaller in size than controls at a similar developmental stage, and this observation is consistent with our previous findings (Kaufman et al., 1989a). Earlier observations on blastocyst diameter in rabbits showed that triploids were consistently smaller than diploids (Bomsel-Helmreich, 1965), although whether this finding bears any relationship to their postimplantation development is unclear. Since blastocyst diameter was not measured in the present study, we are unable to comment on this observation. However, we believe that an analysis of cell number may be a better indication of developmental stage in preimplantation triploid embryos.

It is possible that there is a greater proportion of cells than is normally found in diploid embryos in the trophoderm of the triploid blastocysts and proportionately less in their ICM [which develops into the embryo proper (Gardner, 1972)], and this may partly explain the smaller size of these embryos during the postimplantation period. Certainly the histology of some of the intact decidua appeared to show an abnormally small embryo associated with an excessively large amount of extraembryonic membranes (our unpublished observations; see also Barton et al., 1984; Surani et al., 1987, 1988). An analysis of developmentally advanced blastocysts to establish the proportion of ICM and trophoderm cells would almost certainly be instructive in this regard.

Our study, unlike those of Beatty and Fischberg (1951) and Edwards (1958), is based on large numbers of embryos and consequently allows critical statistical analyses to be performed. We have also been able to

rule out any possible effects of technical (i.e., experimentally induced) stresses on the diandric triploid embryos by using a comparison with appropriate controls. We have shown previously that handling of the embryos, even for just a short period of incubation in tissue culture medium before transferring them to recipients, has an adverse effect on cleavage rate (Henery and Kaufman, 1991). A control was therefore provided that took this aspect into account. Suitable controls were also undertaken to exclude the micromanipulatory procedure as well as electrofusion. Nuclear manipulation is not detrimental to embryonic survival (Evsikov et al., 1990), and, in fact, one recent study on manipulated mouse preimplantation embryos suggested that the drilling of a large opening (rather than a small one) in the zona facilitates hatching of the blastocyst and significantly improves the developmental potential (Cohen and Feldberg, 1991). Finally, we were able to show in an additional control group that a brief incubation in cytoskeletal inhibitors has no obvious detrimental effect on cell number during the preimplantation period (see also Siracusa et al., 1980; Evsikov et al., 1990).

The fusigenic stimulus used in this study was electrofusion. We have found that this is technically superior to the use of inactivated sendai virus for the fusion of karyoplast to cytoplasm if the problems associated with the production and maintenance of sendai virus are taken into account. Also, improved rates of postimplantation development of mouse tetraploids have recently been achieved when electrofusion has been used as a fusigenic stimulus (Kaufman and Webb, 1990) rather than inactivated sendai virus (O'Neill et al.,

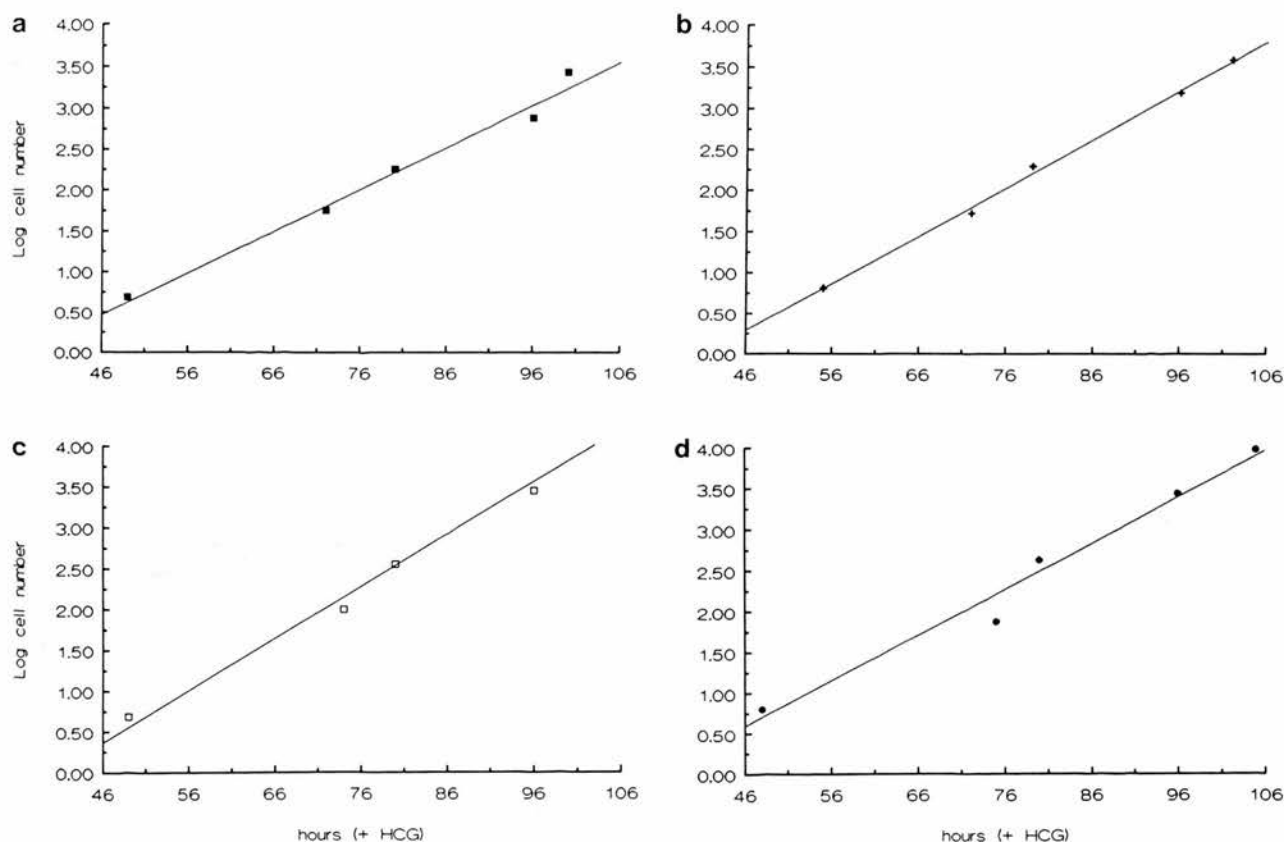


Fig. 1. Log total cell number of embryos isolated at various times during the preimplantation period, plotted against time after HCG injection to induce ovulation in diandric triploid embryos (a), micromanipulated diploid control embryos (b), diploid control embryos exposed to cytochalasin D and colcemid (c), and diploid control embryos (not exposed to cytochalasin D and colcemid) d.

1990). An improved degree of postimplantation development of diandric triploid embryos might, therefore, also be expected to result from the use of this fusigenic stimulus.

Thus we are confident that our results show that the cleavage rate of diandric triploid embryos is not significantly different from that of the various controls used in this study and that their rate of achievement of morula and blastocyst stages also appears to be similar. Furthermore, the high implantation rate observed in the preliminary study (73%) implies that viable blastocysts were formed that were clearly capable of evoking a decidual response. By contrast, most haploid embryos appear to be unable to cavitate normally (Modliński 1975, 1980; Kaufman, 1981, 1983; Henery and Kaufman 1992), but it is unclear whether this is related to cell number (Kaufman and Sachs, 1976) or other factors.

An imbalance in the nucleocytoplasmic ratio of embryos, however, has been shown to have a detrimental effect on their preimplantation development, and this certainly seems to be the case with regard to haploids (Modliński, 1975, 1980; McGrath and Solter, 1986), though it is clearly not the case in relation to the diandric triploids analysed here. The latter have an in-

creased nucleocytoplasmic ratio but cleave and achieve the blastocyst stage at the same time as diploids. It is of interest that Evsikov et al. (1990) increased the nucleocytoplasmic ratio of diploid embryos by the removal of about one-third of the cytoplasm, and this appeared to have the effect of reducing the cleavage rate in this group. However, technical difficulties have been associated with the removal of the female but not the male pronucleus, and this is likely to be the reason why androgenetic haploid embryos produced by micromanipulation develop better than gynogenetic haploids despite the fact that they have an identical nucleocytoplasmic ratio (Modliński, 1975).

While our findings do not exclude the possibility that digynic triploids have a slower cleavage rate (Takagi and Sasaki, 1976) than diandric triploids, they clearly demonstrate that in the mouse triploidy per se is not detrimental to achievement of the blastocyst stage or early postimplantation development. Much information has accumulated over the last few years regarding the different but complementary roles of the maternal and paternal genomes during mammalian embryonic development (McGrath and Solter, 1983, 1984; Barton et al., 1984; Surani et al., 1984, 1986). In the case of the diandric and digynic triploid mouse embryos, however,

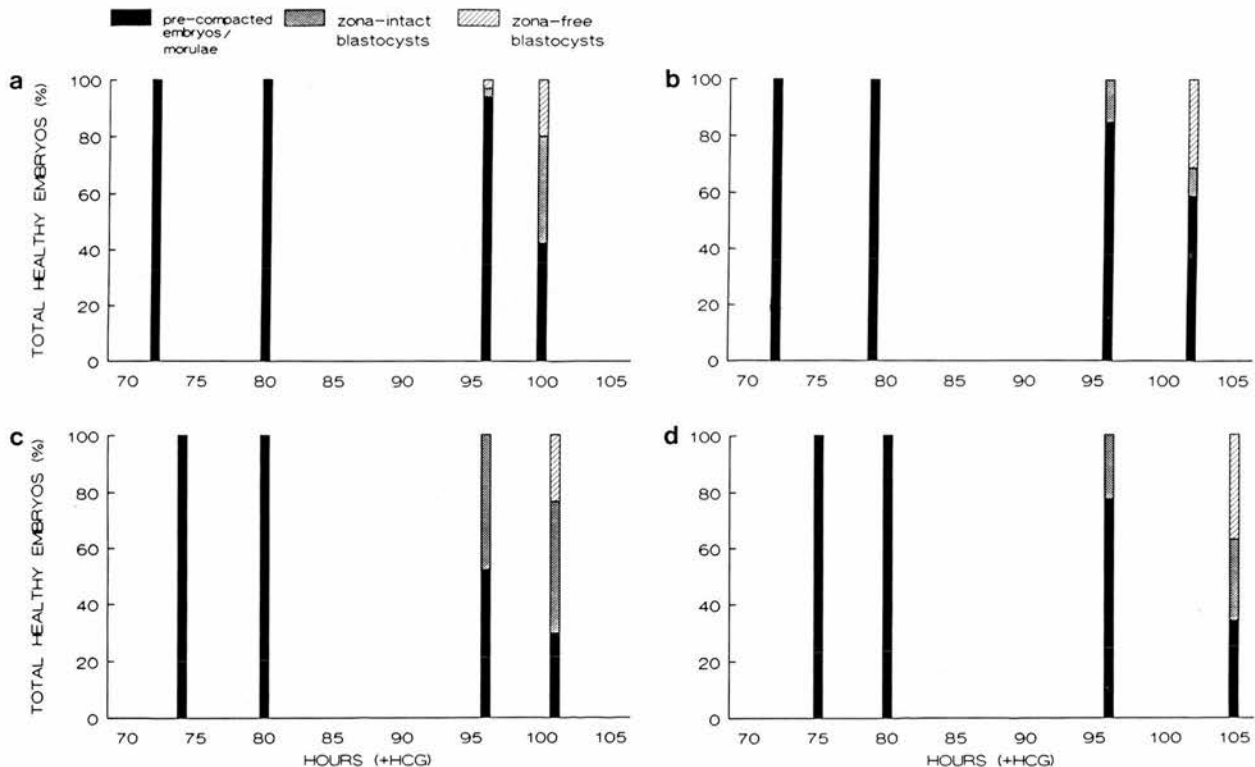


Fig. 2. Morphological appearance of embryos isolated at various times during the preimplantation period, plotted against time after HCG to induce ovulation. a-d refer to the same groups as in Figure 1.

it is unclear how early in development (possibly at some stage during the preimplantation period) the effects of these genomic differences such as imprinting may manifest themselves. Clearly, this is likely to be the explanation for the morphological differences observed between these two classes of embryos during the limb bud stage of development, when the diandric triploids tend to have a normal morphology while the digynic triploids invariably have both neural tube and cardiac defects (Kaufman et al., 1989).

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# Relationship Between Cell Size and Nuclear Volume in Nucleated Red Blood Cells of Developmentally Matched Diploid and Tetraploid Mouse Embryos

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**ABSTRACT** Analysis of control diploid and polyploid amphibia indicated that cell and nuclear volumes were closely related to their ploidy, so that an increase in ploidy was generally associated with an increase in cell size. This relationship is also believed to occur in mammalian polyploids. However, since the latter are only rarely encountered spontaneously, or only occasionally following experimental manipulation, no detailed morphometric studies have been carried out to date to confirm whether such a relationship exists. In this study, the cellular and nuclear volume of primitive red blood cells was analyzed in carefully developmentally matched control diploid mouse embryos and tetraploid embryos produced by the technique of electrofusion. All of the cells and/or nuclei studied had a characteristic spherical shape which greatly simplified the morphometric analysis. A defined and predictable relationship between ploidy and cellular and/or nuclear volume was observed in the red blood cells between 8.25 and 14.5 days of gestation. During this period the primitive red blood cells are gradually replaced by the definitive erythrocytes. The ratio of control values to tetraploid values was found to be close to the theoretical value of 1:2 when comparable cells and/or their nuclei were analyzed in carefully developmentally matched material. © 1992 Wiley-Liss, Inc.

The morphological abnormalities observed in postimplantation tetraploid mouse embryos are believed to be a consequence of an increase in the size of individual cells and a decrease in their overall cell number (Snow, '75). This contention, however, has considerable implications for surface to volume ratios and shape factors not only in tetraploids, but in all higher polyploids (Epstein, '86). That cell size was increased in tetraploids was noted by Snow ('73, '75). Thus, yolk sac-derived blood cells in his tetraploid mouse embryos appeared to be four times greater in volume than comparable cells in diploid controls (Snow, '75), though no detailed morphometric analyses were performed by him to quantify any relationship which might have existed between nuclear and/or cellular volume and ploidy.

A relationship between cell and nuclear volume and ploidy has been proposed in relation to polyploids of recent origin. In general, an increase in ploidy is associated with an increase in cell size, though such a relationship is not commonly observed in long-established polyploid species, where the cellular and nuclear volume tends to decrease towards that characteristic of the diploid parental species (for review, see Beatty, '57). Earlier work on Amphibia found cell or nuclear volume to be directly related to the number of chromosomes present (Fank-

hauser, '45; Fischberg, '48). While cell size was larger, cell number was proportionately reduced, and the overall body size was about the same or often smaller than that of diploids (Fankhauser, '45).

This relationship between cell size and ploidy has not been as fully explored in the higher polyploids of mammals, where examples are only rarely encountered spontaneously, or only occasionally following experimental manipulation have they been induced to develop beyond implantation (for recent review, see Dyban and Baranov, '87). Mammalian liver parenchymal cells have been observed to exhibit a relationship between cell size and ploidy (Epstein, '67). Similarly, cloned tetraploid human fibroblasts formed from diploid cells also appear to show a 2:1 relationship between cell size (and nuclear activity) and ploidy (Chang et al., '83).

A reduction in the overall cell number present in polyploid mammalian embryos/fetuses has been considered to be important in relation both to the morphological abnormalities commonly observed in this group and their poor viability, while the reduction in cell number that also occurs in Am-

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phibia appears to have a less dramatic effect on their viability (see Beatty, '57). Beatty and Fischberg ('51) found that the mean cell number in mouse preimplantation polyploid embryos was approximately inversely related to the number of chromosomes present when such embryos were compared to diploid controls at comparable times after fertilization. A similar relationship was proposed by Edwards ('58) between ploidy and cleavage rate, from his analysis of similar haploid, diploid, and polyploid mouse preimplantation embryonic material. In the pre- and postimplantation mouse tetraploids analyzed by Snow, the cell number was said to be considerably less than in diploids at comparable stages of development (Snow, '73, '75, '76).

A further factor to be considered in relation to the development and differentiation of polyploid embryos/fetuses is the rate of growth of their constituent cells (Edwards, '58). Preimplantation triploid mouse embryos studied by Takagi and Sasaki ('76), for example, were believed to have a cell cycle time approximately 10% longer than that of diploids. This value was estimated from their observation that by the 10th cleavage division (day 5.5) the cell number in their embryos was about half of that of diploid embryos at a comparable stage of development, and that by the 20th cleavage division (day 10.5), they would have had about a quarter of the total number of cells present in a control of the same gestational age. Our own recent findings from the analysis of control and tetraploid preimplantation mouse embryos produced by the technique of electrofusion of blastomeres at the 2-cell stage (Kaufman and Webb, '90; Kaufman, '91) showed that there was no significant difference between the cleavage rates in these two groups (Henery and Kaufman, '91).

Our own study is concerned with attempting to quantify whether a defined relationship exists between cell size and ploidy in developmentally matched groups of normal diploid embryos and homozygous tetraploid embryos. We have quantified cell and nuclear volume using nucleated yolk sac-derived primitive red blood cells in homozygous tetraploid mouse embryos produced by electrofusion and in developmentally matched diploid controls. These cells first enter the embryonic vasculature on day 7.5–8 of gestation and diminish in number during days 14 and 15 (Rugh, '68). These cells, as well as their nuclei, have a characteristic spherical shape throughout their existence in the embryonic circulation, which clearly simplifies any morphometrical analysis. We show that a clear rela-

tionship does indeed exist between cell size and ploidy in these cells.

We also demonstrate that such a relationship is only seen in nucleated red blood cells when material from carefully developmentally matched embryonic stages is compared.

## MATERIALS AND METHODS

### *Control series*

Eight- to 12-week-old randomly cycling female (C57BLxCBA) F1 hybrid mice were caged with (C57BLxCBA) F1 hybrid males. Each morning the females were checked for the presence of a vaginal plug, and the latter was taken as evidence of mating. The morning of finding a vaginal plug was considered to be the first day of gestation.

### *Tetraploid series*

#### **Isolation of 2-cell stage embryos**

Eight- to 12-week-old (C57BLxCBA) F1 hybrid female mice were injected with 5 IU pregnant mares' serum gonadotrophin (PMSG) and then 48 hours later with 5 IU human chorionic gonadotrophin (HCG) to induce ovulation. After the HCG injection, the females were caged individually with fertile homozygous Rb(1.3)1Bnr male mice. The presence of a vaginal plug the next morning was taken as evidence of mating, and this was considered to be the first day of gestation. Early on the morning of the second day of gestation, the female mice were sacrificed by cervical dislocation and the oviducts removed and flushed through with phosphate buffered saline (PBS) containing 4 mg/ml bovine serum albumin, in order to recover the 2-cell stage embryos. These embryos were then transferred into drops of tissue culture medium (Whittingham, '71) under paraffin oil, and retained in an incubator maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> in air.

#### **Electrofusion and transfer of fused tetraploid embryos to pseudopregnant recipients**

The 2-cell stage embryos whose blastomeres were to be fused were transferred into a non-electrolyte solution consisting of 0.3 M Mannitol (Kubiak and Tarkowski, '85), and a similar solution was also present in the fusion chamber. The latter consisted of a plastic tissue culture dish which had two platinum wires each of 250 µm diameter fixed parallel to each other on the bottom of the dish, with a space of about 600 µm between them. The ends of the platinum wires were connected to a digitimer pulse stimulator set at 200 V, with a pulse duration of 50 µsec. The 2-cell stage embryos, in batches



of 10, were then placed between the 2 platinum wires in the chamber and the pulse stimulator was triggered. The embryos were removed immediately and washed through 4 drops of tissue culture medium and then returned to the incubator. Within 15–30 min the 2 blastomeres had fused in a very high proportion of cases to form a single blastomere. These 1-cell (tetraploid) embryos were then transferred unilaterally to the oviducts of recipients on the first day of pseudopregnancy (i.e., on the day of finding a vaginal plug after spontaneously cycling females had been mated to proven sterile vasectomised males). The recipients were anaesthetised with tribromoethanol (Avertin, Winthrop, dose 0.02 ml/g body weight of a freshly prepared 1.2% solution of Avertin dissolved in 0.9% saline).

### ***Analysis of post-implantation stages of development***

The females from the control and tetraploid series were autopsied between the 8th and 15th days of gestation. The decidual swellings were isolated from the uterine horns and put into PBS. The embryos were isolated from within their extra-embryonic membranes and then fixed in Bouin's fixative for subsequent histological examination (Kaufman and Webb, '90). The extra-embryonic membranes of the tetraploid embryos were used to establish their chromosome constitution, using a modification of the technique described by Evans et al. ('72). It was assumed that all of the controls had a normal diploid chromosome constitution.

The embryos fixed for histological examination were embedded in paraffin wax, serially sectioned in the transverse plane, cut at a nominal thickness of 7  $\mu\text{m}$  (embryos up to 11 days p.c.), and at 8  $\mu\text{m}$  for developmentally more advanced embryos, and subsequently stained with haematoxylin and eosin. The exact developmental age of the tetraploid embryos was established by comparing their postcranial morphological/histological features with those of diploid control embryos in the author's (i.e., M.H.K.) reference collection of serially sectioned mouse embryos, based on the morphological staging schemes of Theiler ('89) and Kaufman ('90). In these schemes, somite number, limb bud development, and the degree of differentiation of a wide variety of developing organ systems are compared in order to provide as accurate an assessment as possible of the *developmental* (rather than chronological) age of the tetraploid embryos. The cranial features of the tetraploid embryos were not compared, or included in the analysis, as these were generally grossly abnormal (see Kaufman and

Webb, '90), whereas the histological features of the postcranial viscera were often normal (Kaufman, unpublished).

Mean cellular and nuclear volume was established in nucleated (primitive) red blood cells. These cells were chosen because of the spherical or near spherical nature of their nuclei and their near spherical cellular outline. For spherical cell or nuclear volume the formula  $4/3\pi r^3$  was used to calculate volume,  $r$  being the radius of a nucleus (or cell).

In the cells analyzed, 100 measurements (where possible) were made on the various cells studied in each embryo, using a Kontron/MOP Videoplan image analysing computer linked to a Leitz Laborlux 12 microscope via a Hitachi type 3000 color television camera. All nuclei in the selected fields were measured on sections at not less than 20  $\mu\text{m}$  intervals along the longitudinal axes of the embryos, in order to avoid analyzing individual cells on more than one occasion.

If it is assumed that all nuclei (or cells) within a tissue have a constant spherical or near spherical shape and an average diameter of less than that of the section thickness, then they will be wholly present in the majority of sections studied (Abercrombie, '46). Hence, over 100 measurements, nuclei or cells with a diameter of less than 7  $\mu\text{m}$  or 8  $\mu\text{m}$  (i.e., the section thickness) will give an average unbiased estimate of volume (nuclear or cellular) for that embryo.

By the same argument, it follows that if the cells or nuclei have a diameter of greater than 7  $\mu\text{m}$  or 8  $\mu\text{m}$ , an *underestimate* of cellular or nuclear volume will result. The results will, therefore, show an underestimate of the true volume (cellular and nuclear) for that embryo. In this study, the latter principally applies in relation to the tetraploids. Similarly, in order to avoid measuring the diameters of peripheral sections through primitive erythrocytes when analyzing their cell diameter, only cells in which a nucleus was present in the section were analyzed. Similarly, only cells in interphase were measured, so that cells which were in mitosis were also excluded from this study. The ranges in diameter of primitive red cells and primitive red cell nuclei for the controls were 7.9–10.4  $\mu\text{m}$  and 3.8–6.7  $\mu\text{m}$ , respectively, while for tetraploids the comparable ranges in diameter were 10.1–13.1  $\mu\text{m}$  and 5.0–8.3  $\mu\text{m}$ , respectively.

## **RESULTS**

### ***Comparison of cellular and/or nuclear volume in control diploid and tetraploid embryos***

In this study, serial histological sections from carefully developmentally matched control diploid

TABLE 1. Cellular and nuclear volumes of control diploid and tetraploid primitive red blood cells measured in developmentally matched material between 8.25–14.5 days p.c.

| Developmental stage (days p.c.) | CONTROLS             |                    |                  |                     | TETRAPLOIDS          |                    |                  |                     | Tetraploid/control cell volume | Tetraploid/control nuclear volume |
|---------------------------------|----------------------|--------------------|------------------|---------------------|----------------------|--------------------|------------------|---------------------|--------------------------------|-----------------------------------|
|                                 | No. embryos analyzed | No. cells analyzed | Mean cell volume | Mean nuclear volume | No. embryos analyzed | No. cells analyzed | Mean cell volume | Mean nuclear volume |                                |                                   |
| 8.25                            | 0                    | 0                  | 0                | 0                   | 1                    | 28                 | 690.4            | 254.0               | NA                             | NA                                |
| 8.5                             | 2                    | 200                | 353.2            | 129.8               | 0                    | 0                  | 0                | 0                   | NA                             | NA                                |
| 9                               | 8                    | 800                | 352.5            | 118.2               | 3                    | 300                | 657.8            | 202.9               | 1.87                           | 1.72                              |
| 9.5                             | 2                    | 200                | 390.8            | 119.2               | 3                    | 300                | 675.3            | 216.7               | 1.73                           | 1.82                              |
| 9.75                            | 1                    | 100                | 447.2            | 130.8               | 0                    | 0                  | 0                | 0                   | NA                             | NA                                |
| 10                              | 6                    | 600                | 477.6            | 112.0               | 2                    | 200                | 832.4            | 165.8               | 1.74                           | 1.48                              |
| 10.25                           | 2                    | 200                | 521.0            | 88.3                | 3                    | 300                | 954.8            | 193.7               | 1.83                           | 2.19                              |
| 10.5                            | 1                    | 100                | 525.4            | 92.1                | 1                    | 100                | 1066.8           | 146.2               | 2.03                           | 1.59                              |
| 11.5                            | 1                    | 100                | 484.9            | 54.1                | 1                    | 100                | 1098.7           | 172.1               | 2.27                           | 3.18                              |
| 12                              | 5                    | 500                | 434.0            | 68.6                | 3                    | 300                | 681.3            | 93.9                | 1.57                           | 1.37                              |
| 12.5                            | 2                    | 200                | 384.0            | 36.6                | 1                    | 100                | 706.6            | 86.7                | 1.84                           | 2.23                              |
| 13                              | 2                    | 200                | 371.4            | 40.9                | 0                    | 0                  | 0                | 0                   | NA                             | NA                                |
| 13.5                            | 5                    | 500                | 361.2            | 30.1                | 3                    | 300                | 557.2            | 56.8                | 1.54                           | 1.89                              |
| 14.5                            | 2                    | 200                | 229.2            | 24.7                | 3                    | 300                | 533.1            | 58.2                | 2.33                           | 2.36                              |

and tetraploid embryos were analyzed. Red blood cells were analyzed in this study in order to provide information on cellular and nuclear volume in the developmental period between 8.25 and 14.5 days of gestation.

#### Primitive red blood cellular volume

The mean value for the overall cellular volume of control diploid and tetraploid primitive red blood cells was established by measuring approximately 100 cells from each of a selection of serially sectioned embryos at each time point studied (see Table 1). The ratio of control data to tetraploid data was found to be close to the predicted theoretical ratio of 1:2. The values for controls were found to be significantly different from tetraploid values ( $P < 0.01$ ). The large variation in individual values for cell volume necessitated converting them to natural logarithms as a means of providing data for which a reasonable curve could be produced. These values were plotted and a 5th order polynomial curve was fitted using weighted least squares. The preparation of a curve by this means was chosen, as it provided the best fit for the data. The latter was essential if the curve obtained from the results was to have any predictive value. 95% confidence limits for the curve were calculated and also drawn. Both control and tetraploid regression curves show an early increase in cell volume, reaching a maximum at approximately 10.5–11.5 days of gestation, then a gradual decrease in cell volume until they eventually disappear from the circulation (see Fig. 1).

#### Primitive red blood cell nuclear volume

Corresponding values for primitive red blood cell nuclear volume for the control diploid and tetraploid embryos described above were also obtained from the mean of approximately 100 cells per embryo (see Table 1). These raw values were plotted, regression lines and corresponding 95% confidence limits fitted as indicated above, and the regression lines were found to be significantly different ( $P < 0.01$ ) (see Fig. 2).

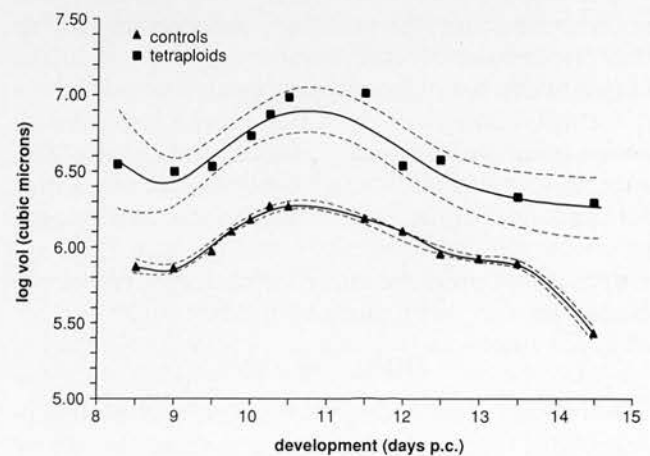


Fig. 1. Regression (5th order polynomial) curves obtained when natural logarithms of the mean values for overall cellular volume obtained from the analysis of control diploid and developmentally matched tetraploid primitive nucleated red blood cells are plotted against developmental age of embryos. 95% confidence limits for each curve are also illustrated (dotted lines). The control and tetraploid regression curves are significantly different ( $P < 0.01$ ).

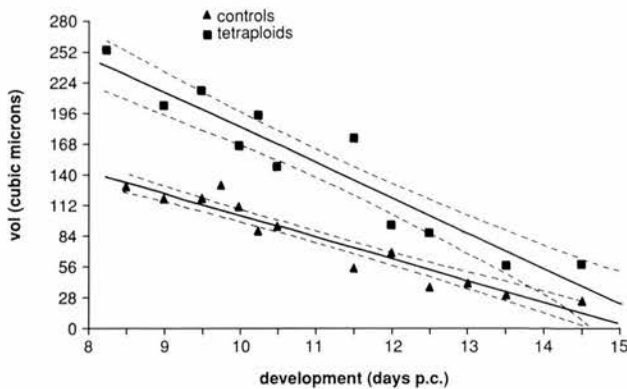


Fig. 2. Regression lines obtained when raw mean values for primitive red blood cell nuclear volumes of control diploid and developmentally matched tetraploid embryos are plotted against developmental age of embryos. 95% confidence limits for each line are also illustrated (dotted lines). The control and tetraploid regression line values are significantly different ( $P < 0.01$ ).

The diploid and tetraploid red blood cells both showed a paralleled (and predictable) decrease in their nuclear volume with increased developmental age. The ratio of control to tetraploid values was again found to be close to the predicted theoretical ratio of 1:2.

A representative selection of photomicrographs of control and tetraploid nucleated primitive red blood cells from developmentally matched embryos at 9 and 10.5 days of gestation are illustrated in Figure 3.

The regression curves and lines for the red blood cellular and nuclear volumes, respectively, fitted over the developmental period from 8.25–14.5 days of gestation, are of important predictive value.

Detailed cytogenetic analyses of the mitoses prepared from the extra-embryonic membranes of tetraploid and diploid control embryos revealed that all the cells studied, in which accurate chromosome counts could be made, had a normal (i.e., euploid) chromosome constitution (Kaufman and Webb, '90; Kaufman and Webb, unpublished).

## DISCUSSION

A relationship between cell size and ploidy in polyploids of recent origin has previously been proposed (Fankhauser, '45; Fischberg, '48; and for reviews, see Beatty, '57; Kaufman, '83; Dyban and Baranov, '87) but has never been quantified, as far as we are aware, in any species by detailed morphometric analysis. Earlier work with amphibian embryos, however, established that there was a direct relationship between cell and/or nuclear volume and the number of chromosomes present.

In the present study, we have observed that both cell and nuclear volume are indeed directly related to ploidy in early postimplantation mouse embryos when comparable cells are studied in developmentally matched diploid and tetraploid embryos. The cell and nuclear volumes of yolk sac-derived primitive red blood cells in tetraploid mouse embryos were found to be almost exactly twice those of comparable diploid cells from developmentally matched control diploid embryos when these were analyzed between 8.25 and 14.5 days of gestation. It is important to stress, however, that this relationship is only seen when comparable cells are analyzed in developmentally matched material, since the absolute volume of a cell and its nucleus can vary considerably depending on a number of factors, such as its functional activity, the stage in the cell cycle, and at different stages during its lifespan (Watson, '77).

That such an increase in cell size does occur in polyploids has previously been observed in relation to the definitive (i.e., non-nucleated) red blood cells of human triploid fetuses (Fisk et al., '89) and in human triploid neonates (Pai et al., '82; Sadowitz et al., '84). Similarly Snow ('73, '75) noted that cell size in tetraploid mouse embryos was increased; and it was suggested that nucleated yolk sac-derived blood cells in these embryos were about 4 times the volume of corresponding diploid cells. Such an increase in cell size has also been reported to occur in human diploid-polyploid mosaic tissues (Kohn et al., '67).

These observations give support to the proposal that in recent polyploids, cell size is in some way related to ploidy. However, as far as we are aware, this is the first detailed study in which morphometric means have been applied to investigate this phenomenon. The findings from our study will be of importance in analyzing the underlying basis of the morphological abnormalities that undoubtedly occur in polyploids, since an increase in cell size and a concomitant decrease in cell number are believed to play a role in the aetiology of the developmental abnormalities that are encountered in these conditions (Epstein, '86).

This relationship between cell size and ploidy will also be of diagnostic importance in those situations where it is necessary to analyze histologically the cellular events that occur in the intact implantation site. Clearly in these instances it would be technically impossible at the same time to analyze the chromosome constitution of the embryos by direct cytogenetic means. Our results are therefore plotted graphically in order to facilitate this exercise. During the developmental period studied, namely



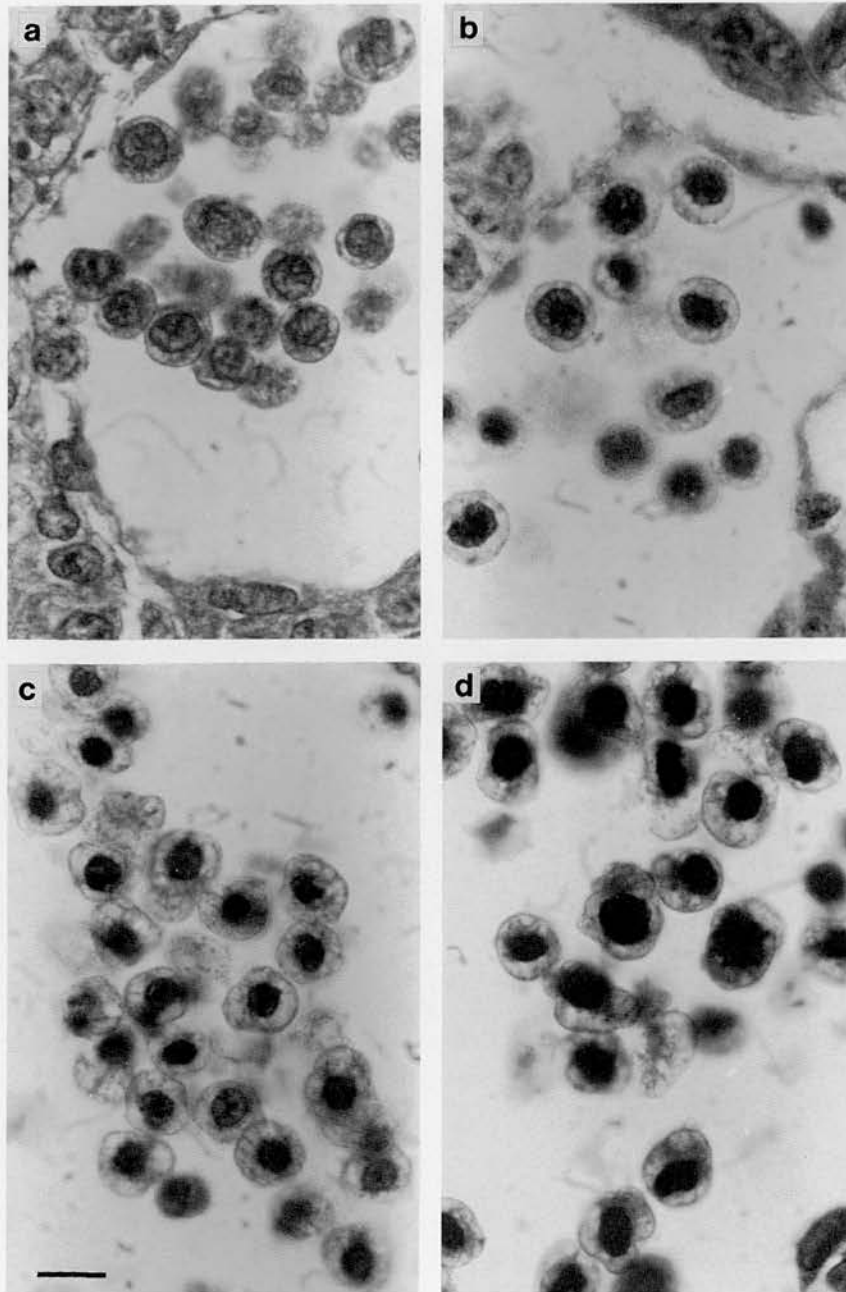


Fig. 3. Photomicrographs of histological sections showing comparable regions within the dorsal aortae of control and developmentally matched tetraploid mouse embryos. All sections are stained with haematoxylin and eosin, and are illus-

trated at the same magnification ( $\times 1,000$ ). Scale bar =  $10\ \mu\text{m}$ . **a, c:** Control embryos at 9 and 10.5 days p.c., respectively. **b, d:** Tetraploid embryos at 9 and 10.5 days p.c., respectively.

between 8.25 and 14.5 days of gestation, red blood cellular and nuclear volume changes in a predictable fashion. After day 11 of gestation, the primitive nucleated red blood cell population is gradually replaced by the definitive erythrocytes which are derived initially from the liver, and later from the spleen and bone marrow. During the overlap period between 11 and 15 days of gestation, however, the

primitive erythrocytes play a diminishing important role, and this coincides with their eventual death and removal from the circulation.

It is unclear why the nuclear and cellular volumes of diploid and tetraploid primitive red blood cells varied to the extent that was observed here. While we cannot exclude the possibility that some of these cells may have lost one or more chromo-

somes during previous divisions, and could account for some small variability in their volume, this possibility seems unlikely, as the cytogenetic analyses of extra-embryonic tissues revealed that these cells always had a normal (i.e., euploid) chromosome constitution. While clearly some of the cells analyzed might have been aneuploid, it was hoped that the analysis of usually between 100 to 600 cells per group would increase the accuracy of the mean values reported for each point studied. The fact that, once differentiated, these cells do not divide again would seem to exclude the possibility that the variation in size seen might be accounted for by the analysis of cells at different stages during the cell cycle.

We plan to analyze other cell types in developmentally matched control and tetraploid embryos to establish whether these, like the primitive red blood cells, change in a predictable fashion over specific periods of time. Other studies are in progress to investigate cellular and nuclear volume relationships in diandric and digynic triploid embryos.

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# Tetraploidy in Mice, Embryonic Cell Number, and the Grain of the Developmental Map

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Tetraploid mice prepared by electrofusion develop for up to 14 days *in utero*. The embryos are essentially normal save that the forebrain and its associated tissues fail to develop properly. Here, we report measurements of cell counts in tissues and volume measurements of tetraploid and control embryos together with observations on the morphology of tetraploid embryos. The results show that the tetraploid embryos are about 85% normal size, but have only a little under half the number of cells of control embryos, with their nuclei being about twice the size of those of diploid cells. Close examination of sectioned material, in contrast, showed that tetraploid morphology and morphogenesis were indistinguishable from those of controls, except in forebrain-associated material. This conclusion gives some insight into an important developmental question, how fine can the developmental map be for normal cellular differentiation to proceed? As tetraploids have only about half the expected number of cells, the ability of these embryos to develop normally in all regions except the forebrain and its derivatives argues that pattern formation mechanisms can cope with the abnormally small number of cells in all regions except the forebrain. The results as a whole argue for size regulation in mammalian embryos being achieved by assaying absolute size rather than counting cell numbers. © 1992 Academic Press, Inc.

## INTRODUCTION

One of the most remarkable properties of many embryos is the extent to which they can regulate their development so that a normal, proportionate animal forms irrespective of its size. Classic examples are Driesch's observation that any one of the first four or even eight sea urchin cells could form a complete if small embryo (1891) and Waddington's demonstration that, if considerable amounts of extra ectoderm were added to a frog gastrula (1938), a normal but large animal would develop. The explanations for this regulative behavior still elude us; indeed the cell-number bounds within which embryos can develop normally are still in general unknown. The answers to the question are, however, important for two reasons: first, it gives some indication as to how size regulation is achieved and, second, it tells us about how pattern formation mechanisms scale to cell number.

Most of the work done in this area has, for obvious reasons, used either invertebrate or amphibian embryos and there have been few opportunities to examine these problems in mammalian embryos. The best-known observations here are those of Tarkowski and of Snow. Tarkowski (1959) showed that destroying one cell of a two- or four-cell mouse embryo gave 11-day embryos of

normal size, while Snow demonstrated that killing about 85% of the cells of a 7-day mouse embryo with mitomycin C had a surprisingly limited effect (Snow and Tam, 1979; Snow *et al.*, 1981; Tam and Snow, 1981). The embryos recovered to a very great extent from this gross insult: gross morphology was essentially normal by 13 days postcoitum (dpc) and many of the animals were born when expected with the only obvious sign of the trauma being a lowered fetal weight. The major abnormalities that resulted were a poor breeding performance and a high incidence of minor axial abnormalities (Gregg and Snow, 1983).

There is, however, an alternative way of approaching the question of how pattern formation mechanisms cope with reduced cell number and that is by increasing cell size while keeping that of the embryo constant, thus reducing the total cell count (Beatty, 1957; Henery and Kaufman, 1991). This can in principle be done by affecting the ploidy of the cells and thus increasing their volume (e.g., Beatty, 1957; Snow, 1973, 1975). In earlier work, we have shown that the volume of nucleated red blood cells of tetraploid mouse embryos is twice that of controls, even though the embryos are much the same size (Henery and Kaufman, 1992). In this paper, we report observations on the cell densities, cell sizes, and number of cells in various tissues in normal mouse embryos and in tetraploid embryos produced by electrofusion. In the discussion, we show that the data imply that these tetraploid embryos have only about half the ex-

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pected number of cells. We then consider the implications of the observations in the context of the one obvious developmental abnormality shown by these embryos, namely a grossly abnormal forebrain.

## MATERIALS AND METHODS

### *Producing Tetraploids*

The technique for producing tetraploids involves electrofusing two two-cell blastomeres and has already been described (Kubiak and Tarkowski, 1985; Kaufman and Webb, 1990; Henery and Kaufman, 1991). In brief, females (C57BL  $\times$  CBA F1 hybrids) were superovulated with hormones, mated with homozygous Rb(1.3)1Bnr males, and the embryos flushed out at the two-cell stage. These were then put into a small chamber containing two parallel platinum wires fixed to the bottom of the dish. A single 50- $\mu$ sec, 200 V pulse was then sufficient to fuse the two blastomeres together. About 12 tetraploid embryos were then transferred to each 1-day pseudopregnant recipient, where they can survive for up to 15 or so days. Their survival rate, however, is not good: only about one or two of these embryos per recipient may, for example, reach 10 days, while the frequency of survival drops as the days pass, with the oldest survivor being isolated at 16 days of gestation. The most advanced tetraploids isolated were found to have a developmental age of between 13.5 and 14.5 dpc. (Kaufman, 1991, 1992; for assessment of developmental age, see Theiler, 1989). Embryos (11.5–14.5 dpc) were analyzed for the work reported here and compared with developmentally matched normal controls. In all cases, cytogenetic analysis of the extraembryonic membranes unequivocally confirmed that these embryos had an homozygous tetraploid chromosome constitution (Kaufman and Webb, 1990).

### *Embryo Size*

Only those tetraploid embryos that possessed a normal, posterianal vertebral axis were analyzed in this study; this comprises about half of those isolated on Days 15–16 of gestation (Kaufman, 1991, 1992). The developmental age of the tetraploid embryos was established by comparing their posterianal morphological/histological features with those of diploid controls (for details, see Kaufman and Webb, 1990; Kaufman, 1991). In general, tetraploids lagged some 24–36 hr behind controls and, in specimens matched for developmental age, were some 10% shorter in terms of crown–rump length.

### *Histology*

Bouin's-fixed control and tetraploid embryos were routinely embedded in paraffin and 8  $\mu$ m transverse se-

rial sections cut, which were then stained with haematoxylin and eosin. The sections were then examined using a Leitz Laborlux 12 microscope with a Hitachi Type 3000 television camera.

### *Morphometric Analysis*

The diameter of cell nuclei was measured from the TV image using a Kontron/MOP Videoplan image-analyzing computer, the program of which automatically calculates nuclear volume. The diameters of all complete nuclei in the selected fields were measured on sections at not less than 20- $\mu$ m intervals along the longitudinal axes of the embryos so as to avoid analyzing individual nuclei twice.

In order to estimate the number of nuclei per unit volume from the numbers per unit area in the tissues analyzed, Aherne's simplified formula (Aherne and Dunhill, 1982) was used since the diameter of the nuclei is less than that of the tissue sections. This formula calculates the number of cells per unit volume ( $N_v$ ),

$$N_v = \frac{2N_A}{i \cdot d + 2t},$$

where  $N_A$  is the number of cells per unit area and  $t$  is the thickness of the sections. To calculate the value of  $i$ , parallel lines with a distance of separation ( $d$ ) equivalent to 20  $\mu$ m were drawn on a transparency that was taped to the TV monitor;  $i$ , the frequency with which nuclei intersect these lines was then measured in selected fields. The values of  $i$  for diploid nuclei was about 0.5 and for tetraploid nuclei about 0.75, a figure that reflects the larger size of the latter's nuclei.

## RESULTS

### *Tetraploid Morphology and Size*

Tetraploid embryos are in many ways normal in appearance at 11 dpc. The only striking difference between them and controls is that the two telencephalic lobes fail to separate normally, and all stages between complete holoprosencephaly (in which only a single cerebral hemisphere is formed) through partial separation of the telencephalic hemispheres are encountered (Kaufman and Webb, 1990). Later, there are clear craniofacial abnormalities involving the forebrain, eyes, and pituitary as well as a characteristic facial appearance (Fig. 1). Lesser abnormalities are found in the aortic arch arteries (Kaufman, 1992) and about half of the developmentally most advanced embryos have an abnormal posterianal vertebral axis (Fig. 1g–1i). In this group, the vertebral abnormalities are invariably associated with the presence of an enormous omphalocele (a large defect in the anterior abdominal wall covered only by a thin

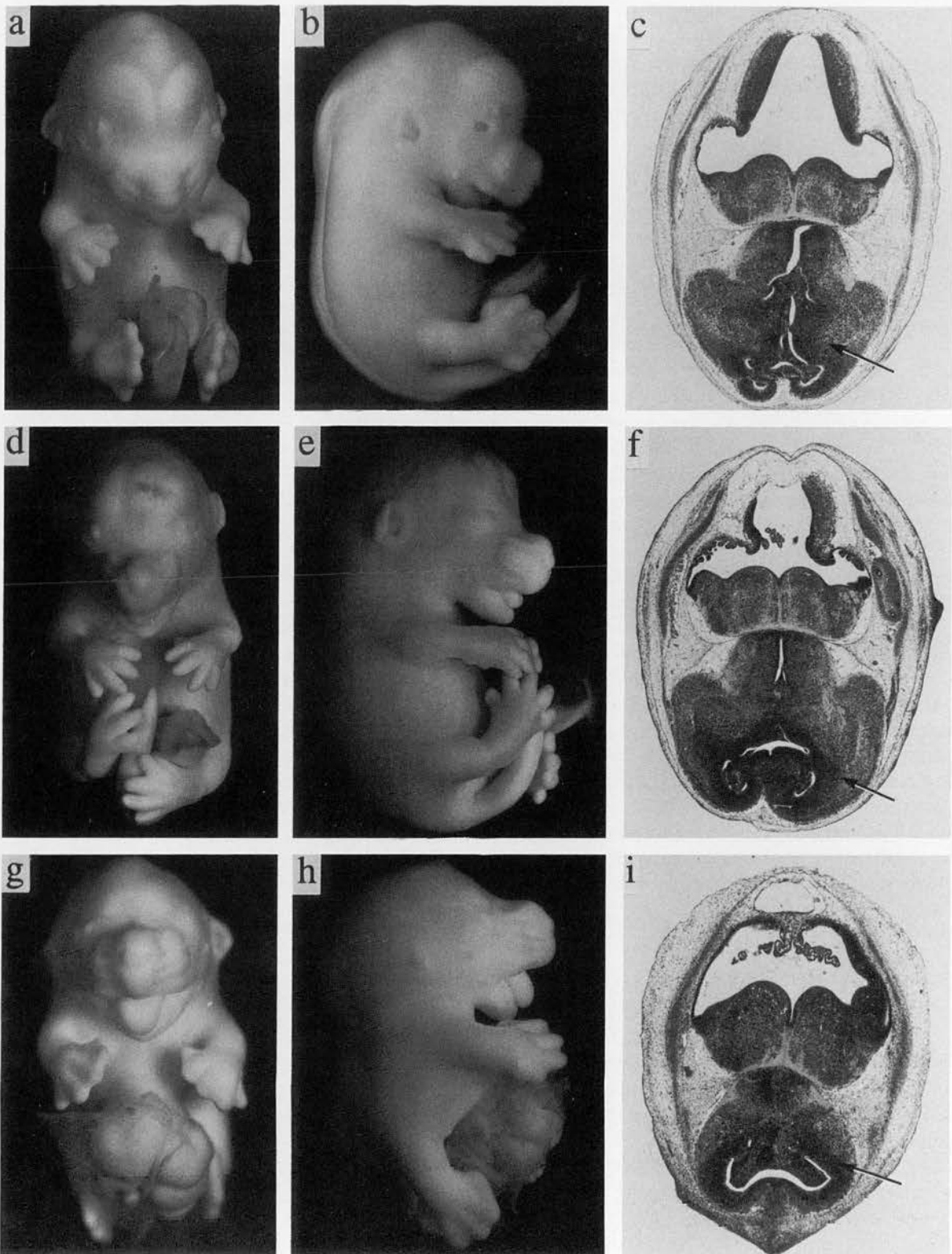


FIG. 1. Frontal and lateral views of three tetraploid embryos, two of which have a normal postcranial vertebral axial morphology (a-c, 13.5 dpc.; d-f, 14.5 dpc.), while a third embryo has an abnormal postcranial vertebral axis associated with an enormous omphalocele (g-i, approximately 14 dpc.). Representative transverse sections through the cephalic region clearly display the grossly abnormal forebrain in these embryos, showing incomplete separation of the two telencephalic hemispheres (arrow; a, b  $\times 6.5$ ; d, e  $\times 5$ ; g, h  $\times 8$ ).

membrane composed of amnion and peritoneum) which contains the abdominal viscera and occasionally the heart. At the histological level, however, the tissues are very similar and tetraploids differentiate as expected. Thus, for example, kidneys develop properly, showing well-developed glomeruli, gonads produce germ cells (Kaufman, 1991), and limb development is normal. Tetraploids are, however, some 10% shorter than normal embryos of equivalent developmental age (Kaufman and Webb, 1990; Kaufman, 1991).

### *Morphometric Measurements*

As it was impractical to measure nuclear size and density in all tetraploid tissues, the tissues selected for analysis were chosen because they could easily be identified in sections and because they reflected a good range of developmental fates. They included cells of the ventral horn of the neural tube (Figs. 2a-2h; 3a and 3b), the developing heart muscle cells (Fig. 3c and 3d), two types of mesenchyme, mandibular connective tissue (Fig. 3e and 3f), and the precartilaginous of the ear (Fig. 3g and 3h). Nuclear size and density within the neural tube was measured in 11.5- to 14.5-day-old tetraploid embryos and the other tissues in 14.5-day-old tetraploids and these were compared with equivalent measurements in controls. As the neural tube is so sharply delineated in transverse sections, its cross-sectional area was also measured (Table 1).

The values for the volumes of tetraploid and diploid cell nuclei are given in Table 2. In the cells of the ventral horn of the neural tube, the ratios of the nuclear volumes over the period 11.5-14.5 dpc remains the same at about 2.34:1. The ratio for the heart-muscle nuclei was a little higher at 2.53:1, while the values for the two connective tissues is a little smaller at about 2.0:1. The estimate of the mean for all the tissues studied is  $2.26 \pm 0.06$ , or a little more than 2.

The numbers of nuclei per field was also counted and this gives a measure of the density of cells per unit area (Table 3). As nuclear diameters are smaller than the thickness of the tissue sections, it is possible to use Aherne's formula to estimate the volume density from the area density (see Materials and Methods). These figures are also given in Table 3 and show clearly that the density of cells in diploid embryos is a little over twice that in tetraploids, with the figures for the ventral horn cells of the neural tube being about 2.25:1, for the heart muscle cells 2.86:1 and the connective tissue mesenchyme about 2:1.

To estimate the ratio of the total numbers of cells in tetraploid and diploid embryos, we require to scale the ratios that are based on tissue sections to the volumes of the whole tissues and embryos. The best figures here are

for the neural tube where the cross-sections are about the same in the two types of embryos, but where the length ratio of tetraploids to diploids is about 0.9:1. Combining the size and cell density ratios gives an estimate of the diploid-to-tetraploid ratio for the total numbers of cells in the neural tube of about 2.5:1. The figures for the mesenchymal tissues are about 2.2:1 and for heart muscle 2.8:1. In short, it seems that the number of cells in tetraploid embryos is only about 40% of that in diploids.

### DISCUSSION

#### *Tetraploid Survival*

Our observations on tetraploid mouse embryos clearly demonstrate that, in each of the tissues studied, there is a severely diminished number of cells that possess doubled-size nuclei. The embryos are, nevertheless, of almost normal size and their only consistently recognizable gross anatomical abnormality is a poorly developed forebrain. Moreover, although plant, fish, and amphibian tetraploids develop quite normally and can reproduce (Fankhauser, 1945; Dawson, 1962; Myers *et al.*, 1986), tetraploid mouse embryos do not survive well: only some 10% achieve hindlimb development (10.5 dpc), and just the occasional embryo reaches day 16 (equivalent to 14-14.5 dpc of normal development). As histogenesis seems quite normal in sectioned material, it is surprising that they die so early. The explanation is probably mundane: tetraploid embryos develop poor placentas and thus have a limited oxygen supply (authors, unpublished), probably due to an imprinting effect (Barton *et al.*, 1985; Moore and Haig, 1991).

In the light of these and our earlier findings, it is hard to interpret the significance of the claim that cytochalasin B-induced, homozygous tetraploid mouse embryos can survive to birth (Snow, 1973, 1975, 1976). Such mice mainly seemed normal if small, although abnormalities involving the forebrain and its derivatives were commonly encountered (e.g., Fig. 1). The difference between Snow's observations and ours could reflect strain variability, but a more likely explanation is that some or possibly all of his most advanced embryos were, in fact, diploid-tetraploid mosaics rather than pure tetraploids, despite the fact that cytogenetic analyses failed to demonstrate this in more than a few instances. His method for making these tetraploids involved exposing two-cell stage embryos to cytochalasin B to inhibit the second cleavage (efficiency ~40-75%). This technique is, however, inherently less reliable than the use of electrofusion to achieve tetraploidy, where only those two-cell embryos used are those that have reverted to a single cell and so have unequivocally diploidized to form homozygous tetraploid embryos.



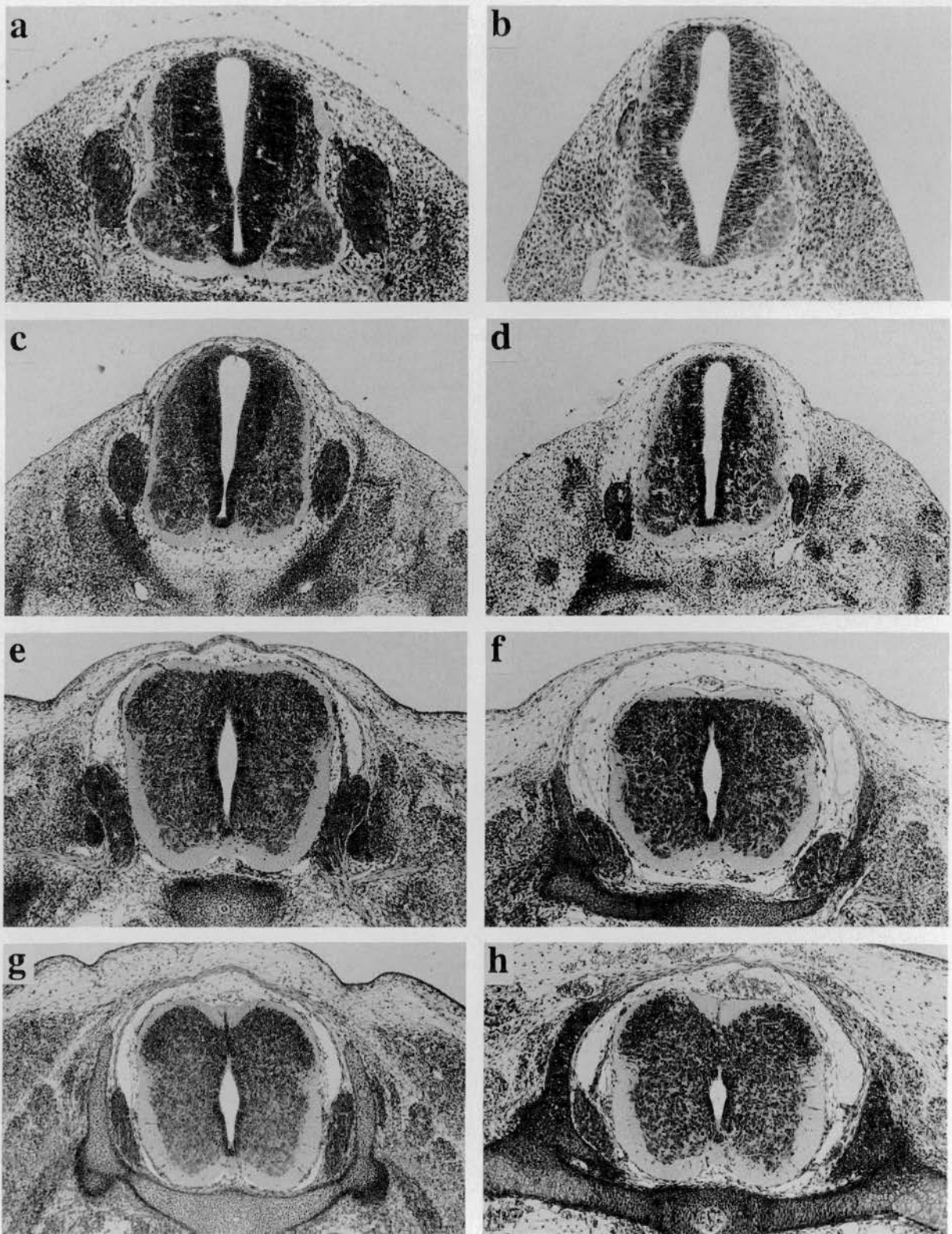


FIG. 2. (a-h) Representative transverse sections through the neural tube (*i.e.*, the developing spinal cord) in the mid-thoracic region in tetraploid embryos (b, d, f, h) and developmentally matched controls (a, c, e, g). a and b, 11.5 dpc; c and d, 12.5 dpc; e and f, 13.5 dpc; g and h, 14.5 dpc. The micrographs demonstrate that there is little if any difference in either the cross-sectional areas of tetraploid and diploid neural tubes or their development (a and b,  $\times 100$ ; c-h,  $\times 63$ ).

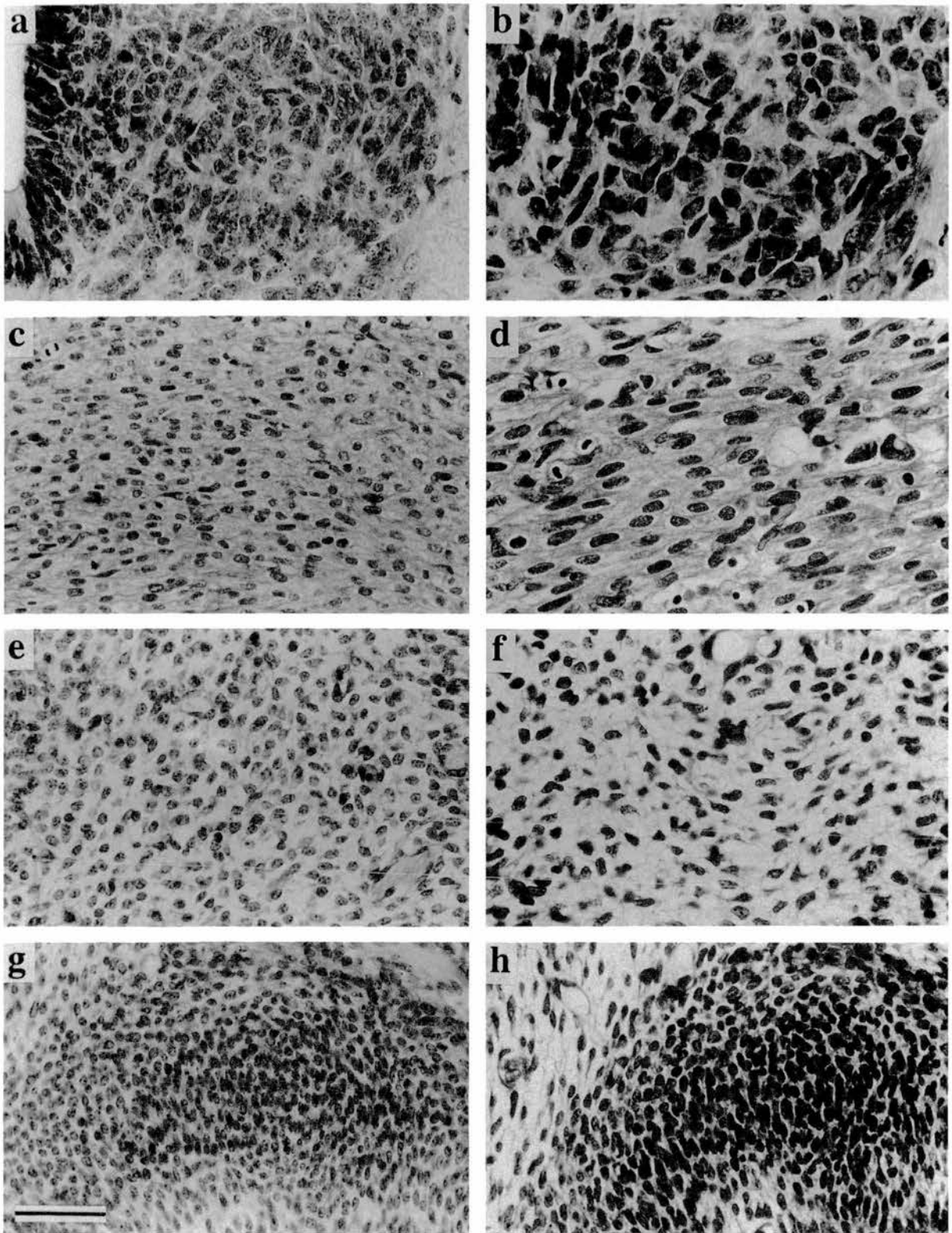


FIG. 3. (a-h) Representative histological sections through a selection of tissues in a 14.5-dpc tetraploid embryo (b, d, f, h) and in a developmentally matched control diploid embryo (a, c, e, g). a and b, cells of the ventral horn of the neural tube; c and d, heart muscle cells; e and f, mandibular mesenchyme tissue; g and h, precartilaginous tissue of the ear. In each of the pairs, both nuclei and cells are clearly larger in tetraploids (right column) than in their diploid controls (left column). (All photomicrographs  $\times 400$ ; bar =  $50\ \mu\text{m}$ ).

TABLE 1  
CROSS-SECTIONAL AREA OF NEURAL TUBE IN TETRAPLOID AND DIPLOID EMBRYOS

| Day of gestation | TETRAPLOID                                     |                        | DIPLOID  |                        | Tetraploid: diploid area ratio |
|------------------|--|------------------------|--|------------------------|--------------------------------|
|                  | Mean neural tube area ( $\mu^2$ ) ( $\pm$ SEM) | Total embryos analyzed | Mean neural tube area ( $\mu^2$ ) ( $\pm$ SEM) | Total embryos analyzed |                                |
| 11.5             | 65872.5 <sup>a</sup>                           | 1                      | 63176.3 ( $\pm$ 4230.7)                        | 2                      | 1.04:1                         |
| 12.5             | 99660.3 ( $\pm$ 11488.9)                       | 3                      | 119347.3 ( $\pm$ 4907.1)                       | 3                      | 0.83:1                         |
| 13.5             | 133027.7 ( $\pm$ 12746.7)                      | 2                      | 130719.8 ( $\pm$ 8832.6)                       | 3                      | 1.02:1                         |
| 14.5             | 147222.9 ( $\pm$ 8315.2)                       | 2                      | 138066.5 ( $\pm$ 5659.8)                       | 3                      | 1.07:1                         |

<sup>a</sup> No SEM as only one embryo was analyzed.

### Regulation Successes and Failures

As with other species (Beatty, 1957), mouse tetraploid tissues have large cells with nuclear volumes about twice the size of their diploid equivalents, irrespective of whether the cells are small (e.g., precartilaginous of the ear and mandibular mesenchyme) or large (heart muscle and ventral horn cells). There are therefore less than half the number of cells in tetraploids as compared to controls. The mechanism by which chromosome complement controls nuclear and cell size in postimplantation embryos remains unknown, but it is clear that regulation is achieved through assaying size rather than by counting cells (e.g., Bard, 1978). The exact stage at which size regulation takes place in mammalian embryos is unclear, but studies on chimeric embryos indicate that it occurs during the early postimplantation period (Tarkowski, 1963; Buehr and McLaren, 1974).

As tetraploid embryos have less than half the number of cells of diploids, it is surprising that the histological

appearance of tetraploid tissues is almost indistinguishable from that of controls. Normal germ cells, ovaries, and testes form (Kaufman, 1991), the fine detail of kidney structure is as expected, and one has to look for such fine detail as the exact pattern of the branchial/pharyngeal arch arteries to find abnormalities in these tetraploids (Kaufman, 1992). Indeed, although there are many fewer cells in these embryos, the coarseness of cellular organization is in general only apparent in cell counts and not in morphology.

The normality of local tissue organization is not matched by the gross appearance of the embryos. Here, the one consistent difference between tetraploids and controls is in the development of the forebrain. In all cases, this reflects incomplete morphogenesis of the two cerebral hemispheres, even though the mid- and hind-brain regions in most instances seem relatively normal. On the basis of their external appearance, the very occasional tetraploid humans that have survived to birth likewise show abnormal forebrain development, tending

TABLE 2  
VOLUMES OF TETRAPLOID AND DIPLOID CELL NUCLEI

| Tissue                       | Tetraploid nuclear volume ( $\mu^3$ ) ( $\pm$ SEM) | Total embryos analyzed | Total cells analyzed | Diploid nuclear volume ( $\mu^3$ ) ( $\pm$ SEM) | Total embryos analyzed | Total cells analyzed | Tetraploid: diploid volume ratio |
|------------------------------|--|------------------------|----------------------|---|------------------------|----------------------|----------------------------------|
| Neural tube                  |  |                        |                      |   |                        |                      |                                  |
| 11.5-day                     | 601.3 <sup>a</sup>                                 | 1                      | 77                   | 241.8 ( $\pm$ 23.0)                             | 2                      | 208                  | 2.49:1                           |
| 12.5-day                     | 491.2 ( $\pm$ 105.6)                               | 3                      | 291                  | 216.3 ( $\pm$ 11.7)                             | 3                      | 327                  | 2.27:1                           |
| 13.5-day                     | 459.1 ( $\pm$ 55.4)                                | 2                      | 177                  | 222.2 ( $\pm$ 20.3)                             | 3                      | 328                  | 2.07:1                           |
| 14.5-day                     | 488.6 ( $\pm$ 54.1)                                | 2                      | 169                  | 192.2 ( $\pm$ 12.3)                             | 3                      | 300                  | 2.54:1                           |
| Precartilaginous of ear      |  |                        |                      |   |                        |                      |                                  |
| 14.5-day                     | 145.4 ( $\pm$ 11.4)                                | 2                      | 215                  | 78.2 ( $\pm$ 4.4)                               | 3                      | 253                  | 1.86:1                           |
| Heart muscle                 |  |                        |                      |   |                        |                      |                                  |
| 14.5-day                     | 300.4 ( $\pm$ 2.1)                                 | 2                      | 164                  | 118.9 ( $\pm$ 20.0)                             | 3                      | 274                  | 2.53:1                           |
| Mandibular connective tissue |  |                        |                      |   |                        |                      |                                  |
| 14.5-day                     | 264.8 ( $\pm$ 45.2)                                | 2                      | 188                  | 126.5 ( $\pm$ 17.0)                             | 3                      | 319                  | 2.09:1                           |

<sup>a</sup> No SEM as only one embryo was analyzed.



TABLE 3  
NUCLEAR DENSITIES FOR TETRAPLOID AND DIPLOID CELL NUCLEI

| TISSUE                       | TETRAPLOID                               |  | DIPLOID                                  |  | $N_V$ Diploid:<br>$N_V$ tetraploid<br>ratio |
|------------------------------|--|--|--|--|---|
|                              | No. cells/area <sup>2</sup><br>( $N_A$ ) | No. cells/vol <sup>3</sup><br>( $N_V$ ) <sup>a</sup> | No. cells/area <sup>2</sup><br>( $N_A$ ) | No. cells/vol <sup>3</sup><br>( $N_V$ ) <sup>a</sup> |   |
| Neural tube                  |  |  |  |  |   |
| 11.5-day                     | 19.2                                     | 1.3  | 34.7                                     | 2.3  | 1.77:1                                      |
| 12.5-day                     | 18.4                                     | 1.2  | 42.0                                     | 2.9  | 2.42:1                                      |
| 13.5-day                     | 25.8                                     | 1.6  | 42.7                                     | 3.1  | 1.93:1                                      |
| 14.5-day                     | 18.9                                     | 1.2  | 43.8                                     | 3.2  | 2.67:1                                      |
| Precartilage of ear          |  |  |  |  |   |
| 14.5-day                     | 53.7                                     | 4.1  | 84.3                                     | 7.1  | 1.73:1                                      |
| Heart muscle                 |  |  |  |  |   |
| 14.5-day                     | 12.7                                     | 0.7  | 27.7                                     | 2.0  | 2.86:1                                      |
| Mandibular connective tissue |  |  |  |  |   |
| 14.5-day                     | 19.5                                     | 1.3  | 35.4                                     | 2.8  | 2.15:1                                      |

<sup>a</sup> These figures are calculated using Aherne's formula (see Materials and Methods).

to have microcephaly, narrow foreheads, microphthalmia, and high-arched and/or cleft palates (Pajares *et al.*, 1990).

That mammalian tetraploids should all have abnormal forebrains and not share any other consistently identifying feature is surprising. The reasons for this abnormality clearly lie neither in growth (the remaining brain, spinal cord, and all other nonneural tissues achieve a normal size), differentiation per se (neurons develop normally), nor the general failure of a morphogenetic process (forebrain development requires no unique morphogenetic mechanisms (Bard, 1990)). Moreover, this fault seems to be limited to mammals: tetraploid trout and amphibia possess brains that appear morphologically and functionally indistinguishable from those of diploids (Myers *et al.*, 1986). It is not easy to see why the mechanisms that underpin tetraploid forebrain differentiation and morphogenesis should go awry in the mammals alone. One obvious possibility, however, is that there are just too few cells in this tissue for the necessary functions to be separately distributed to it by the pattern-formation mechanism that maps mammalian brain development.

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# The Incidence of Aneuploidy After Single Pulse Electroactivation of Mouse Oocytes

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**ABSTRACT** A brief electric pulse often produces a high rate of activation of recently ovulated oocytes. Some other efficient parthenogenetic stimuli, such as alcohol, however, disrupt the spindle apparatus and increase the incidence of aneuploidy. In this paper, we have determined whether electroactivation per se increases the incidence of chromosomal segregation errors in haploid parthenogenones as evidenced at first cleavage mitosis.

Superovulated F1 hybrid female mice were killed at 15.5, 18.5, 22.5, and 25 h after the HCG injection. Batches of 10–12 cumulus-denuded oocytes were transferred to an electroactivation chamber containing mannitol which was connected to a high voltage pulse stimulator and the pulse was triggered once. A high proportion of oocytes activated following this treatment, but only the single-pronuclear haploid parthenogenones were incubated overnight in medium containing colcemid, to determine the incidence of aneuploidy as evidenced at first cleavage mitosis.

"Sham" electroactivation groups were also examined for evidence of activation and aneuploidy as described above. In these cases, cumulus-denuded oocytes were put through the electroactivation chamber but the pulse was not triggered. A further group of oocytes was studied to determine the effect of handling and exposure to hyaluronidase on activation frequency and parthenogenetic pathways. Finally, the spontaneous rate of aneuploidy was examined in fertilised embryos of F1 hybrid female mice  $\times$  Rb(1.3)1Bnr male mice at first cleavage mitosis.

The results show that single pulse electroactivation does not increase the level of aneuploidy in single-pronuclear parthenogenones compared to the "sham" group or the spontaneous rate observed in 1-cell fertilised embryos, nor does aneuploidy appear to increase with postovulatory age. The developmental pathways observed in the electroactivation group are significantly different to those observed in the "sham" group, and the level of activation observed in both groups is increased through handling of oocytes and their exposure to hyaluronidase.

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**Key Words:** Aneuploidy, Electroactivation, Single pulse, Parthenogenesis, Mouse

Kaufman, 1983). Their development can be initiated both spontaneously and experimentally by exposing unfertilised oocytes to a variety of activating stimuli (Kaufman, 1983). Parthenogenones are useful for studying the influence of ploidy and parental genotypes on development.

The exposure of oocytes to pulses of electricity appears to be an effective activating stimulus in mice (Tarkowski et al., 1970; Witkowska, 1973 a,b; Gulyas, 1976; Onodera and Tsunoda, 1989; Didion et al., 1990; Landa and Hajkova, 1990; Marcus, 1990; Rickords and White, 1992; Vitullo and Ozil, 1992), rabbits (Gulyas, 1976; Ozil, 1990; Fissore and Robl, 1992), hamsters (Kaufman et al., 1975; Gulyas, 1976), pigs (Prather et al., 1989) and cattle (Ware et al., 1989). This stimulus is also widely used in nuclear transplantation studies to fuse either a donor blastomere or nucleoplast fragments to a recipient oocyte and at the same time activate the latter (Stice and Robl, 1988; Collas et al., 1989; Robl and Stice, 1989). Recently, this experimental procedure has been used to simulate events at fertilisation, in which intracellular  $\text{Ca}^{2+}$  oscillations occur, and the level of electrical stimulation employed has been found to have a significant effect on the development potential of the resultant parthenogenones (Ozil, 1990).

It is relevant to establish whether aneuploidy may occur as a direct or indirect consequence of electrical stimulation of oocytes. Aneuploidy can result from the malsegregation of chromosomes at either the first or second meiotic division, and it is thought that in man maternal meiotic errors are four times as common as paternal errors (Chandley, 1986). It occurs spontaneously at a low rate in mice (about 1%, Dyban and Baranov, 1987), but its incidence can increase dramatically following exposure of oocytes to spindle disrupting drugs such as ethanol and general anaesthetics (Kaufman, 1977, 1982; O'Neill and Kaufman, 1988).

The brief exposure of oocytes to a dilute solution of ethanol is a particularly effective activating stimulus (Kaufman, 1982; Cuthbertson, 1983; O'Neill and Kaufman, 1989), but a relatively small (10–20%), nevertheless, a significant proportion of the resultant par-

## INTRODUCTION

Parthenogenetic embryos develop without any contribution from the male gamete (Beatty, 1957;

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thenogenones are found to be aneuploid (Kaufman, 1982; O'Neill and Kaufman, 1989; O'Neill et al., 1989). Strontium chloride is also an effective activating stimulus but, unlike ethanol, induces relatively low rates of aneuploidy (O'Neill et al., 1991).

Changes associated with postovulatory aging may also lead to an increase in the incidence of chromosomal segregation errors at the second meiotic division in fertilised embryos (Eichenlaub-Ritter et al., 1986), but this does not appear to be an invariable finding in relation to either *in vivo* or *in vitro* aged mouse oocytes (Donahue and Karp, 1973; Badenas et al., 1989). This is also the case when postovulatory aged mouse oocytes are activated parthenogenetically with hyaluronidase (O'Neill and Kaufman, 1988). These observations would seem to indicate that neither parthenogenesis *per se*, nor postovulatory aging of the oocyte is invariably associated with aneuploidy. However, postovulatory aging of oocytes prior to their fertilisation is associated with an increased incidence of polyploidy (Vickers, 1969; Badenas et al., 1989).

It is well documented that postovulatory aged oocytes activate more readily than do recently ovulated oocytes (Kaufman, 1973; Webb et al., 1986; O'Neill and Kaufman, 1988; Shaw and Trounson, 1989). The postovulatory age of the oocyte at the time of activation also controls the incidence of the various classes of parthenogenone induced (Kaufman, 1973; Kaufman and Surani, 1974), and this is most likely to be a consequence of the age-related changes that occur in the location of the meiotic spindle apparatus and the cortical granules, and the state of polymerisation of the cytoskeletal elements (Szöllösi, 1971, 1975; Longo, 1980). The retention of both products of the second meiotic division may lead to the production of diploid parthenogenones, and a higher proportion of such embryos develop to the blastocyst stage and into the early postimplantation period than haploid parthenogenones (Henery and Kaufman, 1992).

Since over the last few years electrical stimulation has become increasingly widely used for the production of parthenogenones, it is of interest to analyse in detail the immediate cytogenetic response of cumulus-denuded oocytes to a constant pulse of electrical stimulation. The use of a single pulse activation stimulus contrasts with a recent preliminary study of one-pronuclear haploid parthenogenones that resulted from repetitive pulse stimuli involving recently ovulated oocytes (Vitullo and Ozil, 1992). It was also unclear from this report whether the low level of aneuploidy observed was due to hypo- or hyperhaploidy. We have therefore investigated whether electrical pulses *per se* have any effect on the incidence of aneuploidy at a variety of postovulatory ages.

## MATERIALS AND METHODS

### The Electroactivation of Cumulus-Free Oocytes

Eight- to 12-week-old (C57BL × CBA)F1 hybrid female mice were injected with 5 IU pregnant mares'

serum gonadotrophin (PMSG) followed 48 h later by 5 IU human chorionic gonadotrophin (HCG). At 15.5, 18.5, 22.5, and 25 h after the HCG injection, the mice were killed by cervical dislocation. Their oviducts were dissected out, and the cumulus masses containing the ovulated oocytes were released from the ampullary region of the oviduct into phosphate-buffered saline (PBS). The cumulus masses were transferred to microdrops of M16 tissue culture medium (Whittingham, 1971) containing 1 mg/ml of hyaluronidase equilibrated in an atmosphere of 5% CO<sub>2</sub> in air at 37°C for 2–3 min, to remove the cumulus cells. The cumulus-denuded oocytes were then washed four times in M16.

Batches of 10–12 oocytes were then transferred into a nonelectrolyte solution consisting of 0.3M mannitol; a similar solution was also present in the electroactivation chamber. The latter consisted of a plastic tissue culture dish which had two platinum wires each of 250 µm diameter fixed parallel to each other on the bottom of the dish with a space of about 600 µm between them. The ends of the platinum wires were connected to a digitimer pulse stimulator set at 110 mV, 130 mA, with a pulse duration of 50 µs. The batches of oocytes were placed between the wires and the pulse stimulator was triggered. The embryos were removed immediately and washed through four drops of M16 medium and returned to the incubator for 5–6 h.

After this time, four classes of parthenogenone could be determined: (1) oocytes containing a single (haploid) pronucleus, having previously extruded a second polar body (1PN); (2) oocytes containing two (haploid) pronuclei in the absence of second polar body extrusion (2PN); (3) oocytes that underwent "immediate cleavage" in which two equal-sized blastomeres had formed, each containing a single (haploid) pronucleus, one of the blastomeres representing the second polar body (IC); and (4) oocytes in which a single (diploid) pronucleus developed in the absence of second polar body extrusion (1PND).

Only the single-pronuclear haploid parthenogenones (1PN) were studied further. They were subsequently transferred 10 h after activation to microdrops of M16 culture medium containing 1 µg/ml of colcemid in order to arrest their development at metaphase of the first cleavage division. Early the next morning, chromosome spreads were prepared by the air-drying technique described by Tarkowski (1966) and stained in 10% Giemsa at pH 6.8. The chromosome constitution of each preparation was then determined under an oil immersion objective.

### Control Group

#### Sham electroactivation of cumulus-free oocytes.

In this group, F1 hybrid mice were superovulated as described above, and at 15.5, 18.5, 22.5, and 25 h after the HCG injection were killed by cervical dislocation. The cumulus masses were recovered as described above and were transferred into microdrops of previously equilibrated M16 medium containing 1 mg/ml of hyalu-



TABLE 1. Effect of Electroactivation on the Chromosome Constitution of Single-Pronuclear Haploid Parthenogenones at Metaphase of the First Cleavage Division

| Group | Postovulatory age (h) | No. of chromosome spreads | No. of spreads analysable | Chromosome constitution |    |     |    |    | Adjusted rate aneuploidy (%) |
|-------|-----------------------|---------------------------|---------------------------|-------------------------|----|-----|----|----|------------------------------|
|       |                       |                           |                           | 18                      | 19 | 20  | 21 | 22 |                              |
| 1     | 15.5                  | 192                       | 170                       | 3                       | 10 | 156 | 0  | 1  | 1.2                          |
| 2     | 18.5                  | 375                       | 297                       | 2                       | 32 | 257 | 5  | 1  | 4.0                          |
| 3     | 22.5                  | 243                       | 197                       | 3                       | 9  | 184 | 1  | 0  | 1.0                          |
| 4     | 25.0                  | 210                       | 164                       | 1                       | 12 | 149 | 2  | 0  | 2.4                          |

ronidase for 2–3 min, in order to remove cumulus cells. The oocytes were then washed four times in M16 medium.

Batches of 10–12 oocytes were then transferred into a tissue culture dish containing nonelectrolyte solution and then into the electroactivation chamber. No pulse was triggered, and the oocytes were removed and washed four times in M16 medium and returned to the incubator for 5–6 h. Four classes of parthenogenone were observed. The single-pronuclear parthenogenones were further cultured in M16 medium containing 1 µg/ml of colcemid and early the next morning chromosome spreads were prepared and stained as described above.

**Handling of oocytes.** F1 hybrid mice were superovulated and the cumulus masses recovered at the same times after the HCG injection as in the experimental series. Their cumulus cells were removed and activation rate determined after 5–6 h.

**Fertilised embryos.** An additional group of F1 hybrid mice were superovulated and caged individually with fertile homozygous Rb(1.3)1Bnr male mice immediately after the HCG injection. The genotype of homozygous Rb(1.3)1Bnr males (2n = 38) contains two large metacentric chromosomes, being Robertsonian translocations involving chromosomes 1 and 3. Consequently, following fertilisation by spermatozoa from these males, the paternally derived haploid genome contains 18 acrocentric and one large metacentric "marker" chromosome. Thus, these diploid fertilised embryos will contain a normal female chromosome complement (n = 20) and a male complement (n = 19) that contains a "marker" chromosome. The presence of the marker in a first cleavage mitotic metaphase spread that contains two distinct groups of chromosomes allows the female and male chromosome sets to be distinguished.

Early the next morning, the females were examined for the presence of a vaginal plug, and this was taken as evidence of mating. At 6–8 h after the expected time of fertilisation, the females were killed by cervical dislocation, their oviducts were isolated, and their cumulus masses were released into microdrops of M16 medium containing 1 mg/ml of hyaluronidase. These were retained in the latter for 2–3 min to remove the cumulus cells and then washed in four changes of M16. The presence of two pronuclei and a second polar body in the fertilised group was evident at this time. The fertilised embryos were isolated, and only these were returned to

the incubator for a further 5–6 h. First cleavage chromosome spreads were prepared and analysed as described earlier.

A small proportion of embryos could not be successfully analysed due either to overlapping of the chromosomes in the spread, to the fact that the male and female chromosome sets were indistinguishable, or due to scattering of chromosomes, and in a few cases because interphase nuclei were present.

To accommodate for the fact that chromosomes may be lost for technical reasons, and this may inadvertently increase the level of hypohaploid preparations present, an adjusted rate of aneuploidy was used. The formula for this was

$$\frac{\text{No. hyperhaploid spreads}}{\text{total No. haploid spreads analysable}} \times 2 = \text{adjusted incidence of aneuploidy}$$

### Statistical Analysis

Chi-square tests were used in this study. The analysis of the chromosome constitution of the embryos was done using the Fisher exact probability test. The significance value was  $P = 0.05$ .

## RESULTS

### Incidence of Aneuploidy

**Effect of electroactivation on the chromosome constitution of single-pronuclear haploid parthenogenones at metaphase of the first cleavage division.** The effect that electroactivation has on the chromosome constitution of single-pronuclear embryos is shown in Table 1. At 15.5, 18.5, 22.5, and 25 h, the adjusted frequency of aneuploidy is 1.2, 4.0, 1.0, and 2.4%, respectively. The results obtained from the chromosome analysis of embryos that were not exposed to the electric pulse are shown in Table 2. At comparable times to the former group, the adjusted frequencies were 0, 2.8, 3.2, and 3.2%, respectively. Thus, there was no significant difference in the incidence of aneuploidy between the experimental group of single-pronuclear haploid parthenogenones and the control group of parthenogenones ( $P > 0.05$ ). These results demonstrate that electroactivation in vitro does not increase the incidence of chromosomal segregation errors as evidenced at the first cleavage mitosis.

**Chromosome constitution of in vivo fertilised embryos at metaphase of the first cleavage divi-**

**TABLE 2. Chromosome Constitution of Single-Pronuclear Haploid Parthenogenones at Metaphase of the First Cleavage Division after Control "Sham" Electroactivation**

| Group | Postovulatory age (h) | No. of chromosome spreads | No. of spreads analysable | Chromosome constitution |    |     |    |    | Adjusted rate aneuploidy (%) |
|-------|-----------------------|---------------------------|---------------------------|-------------------------|----|-----|----|----|------------------------------|
|       |                       |                           |                           | 18                      | 19 | 20  | 21 | 22 |                              |
| 1     | 15.5                  | 36                        | 33                        | 0                       | 1  | 32  | 0  | 0  | 0.0                          |
| 2     | 18.5                  | 257                       | 217                       | 4                       | 13 | 197 | 2  | 1  | 2.8                          |
| 3     | 22.5                  | 291                       | 186                       | 1                       | 8  | 174 | 3  | 0  | 3.2                          |
| 4     | 25.0                  | 303                       | 190                       | 3                       | 13 | 171 | 2  | 1  | 3.2                          |

**TABLE 3. Chromosome Constitution of In Vivo Fertilised Embryos (F1 hybrid × Rb(1.3) 1Bnr) at Metaphase of the First Cleavage Division**

| Group                         | No. of analysable preparations <sup>a</sup> | Chromosome constitution |     |     |    |    | Adjusted rate aneuploidy (%) |
|-------------------------------|---|-------------------------|-----|-----|----|----|------------------------------|
|                               |   | 18                      | 19  | 20  | 21 | 22 |                              |
| Female (F1 hybrid) pronucleus | 330   | 3                       | 11  | 315 | 1  | 0  | 0.6                          |
| Male (Rb(1.3)1Bnr) pronucleus | 326   | 17                      | 303 | 6   | 0  | 0  | 3.7                          |

<sup>a</sup>The total number of fertilised 1-cell stage embryos examined was 582, 6 of which were triploid.

**TABLE 4. The Effect of Electroactivation on Parthenogenetic Activation Rates and Developmental Pathways in Relation to Postovulatory Age**

| Group | Postovulatory age (h) | No. of oocytes recovered | No. of oocytes activated | Activation rate (%) | Developmental pathways <sup>a</sup> |     |    |      |
|-------|-----------------------|--------------------------|--------------------------|---------------------|-------------------------------------|-----|----|------|
|       |                       |                          |                          |                     | 1PN                                 | 2PN | IC | 1PND |
| 1     | 15.5                  | 406                      | 234                      | 57.6                | 192                                 | 15  | 19 | 8    |
| 2     | 18.5                  | 439                      | 398                      | 90.7                | 375                                 | 11  | 9  | 3    |
| 3     | 22.5                  | 316                      | 304                      | 96.2                | 243                                 | 41  | 15 | 5    |
| 4     | 25.0                  | 644                      | 543                      | 84.3                | 382                                 | 76  | 84 | 1    |

<sup>a</sup>1PN, single-pronuclear haploid parthenogenone; 2PN, 2 pronuclear (diploid) parthenogenone; IC, immediate cleavage parthenogenone; 1PND, single-pronuclear diploid parthenogenone. For further details, see text.

**TABLE 5. Parthenogenetic Activation Rates and Developmental Pathways After "Sham" Electroactivation in Relation to Postovulatory Age**

| Group | Postovulatory age (h) | No. of oocytes recovered | No. of oocytes activated | Activation rate (%) | Developmental pathways <sup>a</sup> |     |    |      |
|-------|-----------------------|--------------------------|--------------------------|---------------------|-------------------------------------|-----|----|------|
|       |                       |                          |                          |                     | 1PN                                 | 2PN | IC | 1PND |
| 1     | 15.5                  | 988                      | 72                       | 7.3                 | 67                                  | 5   | 0  | 0    |
| 2     | 18.5                  | 729                      | 273                      | 37.4                | 257                                 | 15  | 0  | 1    |
| 3     | 22.5                  | 462                      | 331                      | 71.6                | 291                                 | 38  | 0  | 2    |
| 4     | 25.0                  | 709                      | 488                      | 68.8                | 410                                 | 52  | 23 | 3    |

<sup>a</sup>See Table 4.

**sion.** Out of 336 maternally derived chromosome preparations that were analysed in this study, only 1 was hyperhaploid and this gave an adjusted aneuploidy rate of 0.61% (Table 3). Most of the aneuploid preparations encountered were hypohaploid, but the majority probably represent technical artifacts; 1.8% of fertilised embryos were triploid (one diandric, three digynic, and two triploids of unknown origin).

#### Activation Rates

**The effect of electroactivation on the rate of parthenogenetic activation.** The activation rate of oocytes recovered at 15.5, 18.5, 22.5, and 25 h after the HCG injection and subjected to an electrical pulse is

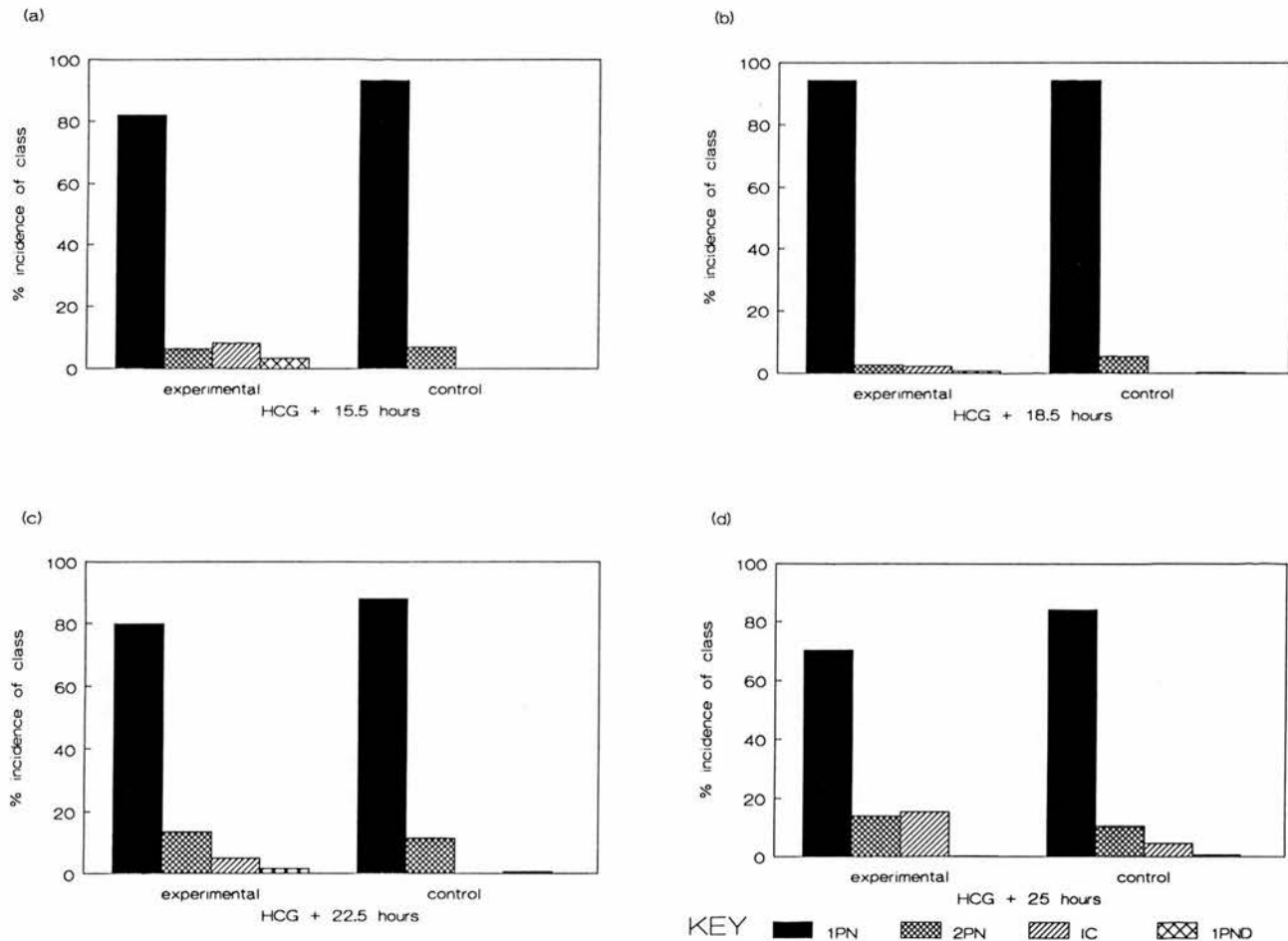
shown in Table 4. The activation rates of the oocytes not exposed to an electrical pulse in the sham electroactivation groups are shown in Table 5. In the 15.5-h group, the level of activation is approximately eight times higher in oocytes exposed to an electrical pulse than in those which were not (57.6% vs. 7.3%), and this difference is highly significant ( $P < 0.001$ ). At all other time points, the activation rate of oocytes in the experimental group is significantly higher than the control group ( $P < 0.001$ ).

**The effect of handling and exposure to hyaluronidase on the rate of parthenogenetic activation.** The activation rate of oocytes in response to handling and exposure to hyaluronidase increased over



**TABLE 6. Parthenogenetic Activation Rates and Developmental Pathways After Handling and Exposure to Hyaluronidase**

| Group | Postovulatory age (h) | No. of oocytes recovered | No. of oocytes activated | Activation rate (%) | Developmental pathways <sup>a</sup> |     |    |      |
|-------|-----------------------|--------------------------|--------------------------|---------------------|-------------------------------------|-----|----|------|
|       |                       |                          |                          |                     | 1PN                                 | 2PN | IC | 1PND |
| 1     | 15.5                  | 101                      | 0                        | 0                   | 0                                   | 0   | 0  | 0    |
| 2     | 18.5                  | 139                      | 13                       | 9.4                 | 12                                  | 1   | 0  | 0    |
| 3     | 22.5                  | 57                       | 17                       | 29.8                | 16                                  | 1   | 0  | 0    |
| 4     | 25.0                  | 95                       | 65                       | 68.4                | 49                                  | 14  | 2  | 0    |

<sup>a</sup>See Table 4.

**Fig. 1.** Histograms demonstrating the proportionate incidence of the various developmental pathways of parthenogenetic embryos observed in relation to postovulatory age after electroactivation (experimental) and "sham" electroactivation (control). **a-d:** HCG + 15, +18.5, +22.5 and +25 h, respectively. 1PN, single-pronuclear haploid parthenogenone; 2PN, 2 pronuclear (diploid) parthenogenone; IC, immediate cleavage parthenogenone; 1PND, single-pronuclear diploid parthenogenone.

time and these findings are shown in Table 6. The activation rate of oocytes recovered at HCG + 15.5, 18.5, 22.5, and 25 h was 0, 9.4, 29.8, and 68.4%, respectively.

#### The Effect of Electroactivation on Parthenogenetic Developmental Pathways

The incidence of the various pathways of parthenogenetic development observed after isolation of the oocytes at HCG + 15.5, 18.5, 22.5, and 25 h both in the

experimental and in the control groups is shown in Figure 1. The highest percentage of activated oocytes in both groups belonged to the single-pronuclear haploid parthenogenetic class (1PN). A comparison of the pathways observed at the various time points studied in oocytes activated by an electrical pulse and those which were not revealed that there was a significant difference between them ( $P < 0.05$ ). Representatives of all of the four classes of parthenogenones were always found

in the experimental groups, but this finding was only observed in the controls in the HCG + 25 h group, with the occurrence of some IC embryos. At this time point, only 70% of the parthenogenones are of the 1PN class in the experimental group, whereas 84% of the control group are of this type. It appears therefore, that the electrical stimulus induces oocytes to progress along different developmental pathways to those normally observed in the control group.

### Effect of Postovulatory Aging on Parthenogenetic Development

The activation rate of oocytes isolated at HCG + 15.5 h was significantly lower ( $P < 0.001$ ) than that of oocytes isolated at HCG + 18.5, 22.5, and 25 h both in the experimental and in the control groups. In addition, the activation rate of oocytes isolated at HCG + 18.5 h was significantly different from oocytes isolated at 22.5 h in both groups ( $P < 0.01$ ). The activation rate increased with the postovulatory age of the oocyte, and peak activation rates were achieved in both groups at HCG + 22.5 h, after which time the activation rates decreased. No significant difference was observed in the incidence of aneuploidy in relation to increasing postovulatory age of the oocyte in either the experimental or the control groups (Tables 1 and 2).

### DISCUSSION

Unfertilised oocytes can undergo parthenogenetic development following activation by a wide range of physical or chemical stimuli (Kaufman, 1983). Electroactivation is a successful stimulus and is widely used in experiments designed to clone animals. The electric pulse used to fuse a donor nucleus to a recipient oocyte will also often simultaneously activate that oocyte and enable reprogramming of the donor nucleus as well as stimulating its subsequent development (Stice and Robl, 1988; Robl and Stice, 1989). It is also a useful stimulus in that it simulates events that normally occur at fertilisation. In the mouse and hamster, the activation stimulus provided by the fertilising sperm produces (among other events) an elevation in intracellular  $\text{Ca}^{2+}$  levels followed by further smaller pulses of  $\text{Ca}^{2+}$  (Cuthbertson et al., 1981; Cuthbertson and Cobbold, 1985; Miyazaki, 1988). It is also clear that the resumption of meiosis and cortical granule exocytosis is dependent on this intracellular rise in  $\text{Ca}^{2+}$  (Kline and Kline, 1992). Most parthenogenetic stimuli only produce a single large but transient increase in intracellular  $\text{Ca}^{2+}$ . It has recently been reported that repetitive pulse stimulation (as opposed to the production of a single pulse) in medium containing  $\text{Ca}^{2+}$  can very closely simulate the intracellular ionic oscillations that normally occur at fertilisation (Ozil, 1990; Vitullo and Ozil, 1992).

Some effective parthenogenetic agents such as ethanol can, however, induce the production of high rates of aneuploidy in the resultant embryos (Kaufman, 1982; O'Neill and Kaufman, 1989), and we were interested to establish whether a single electrical activation pulse

had a similar effect. Our findings reported here would seem to unequivocally establish that no significant difference in the incidence of aneuploidy is observed between oocytes that were electrically activated and those in the control groups at any of the postovulatory ages analysed. Clearly, the present electroactivation and postovulatory aging study (see also O'Neill and Kaufman, 1988) indicates that these factors have little effect on the induction of aneuploidy, although it has been suggested that postovulatory aging of oocytes per se in humans may be the cause of the increased incidence of meiotic errors observed in fertilised embryos (German, 1968). In mice, however, this does not appear to be the case (Donahue and Karp, 1973; Badenas et al., 1989). Vitullo and Ozil (1992) in a much smaller study of electrically activated single-pronuclear haploid parthenogenones to that reported here, noted that at HCG + 12 h, 4 of 59 activated oocytes (i.e., 7%) displayed evidence of aneuploidy, though no indication was given whether this was due to hypo- or hyperhaploidy. Since it is now well established that poor spreading of preparations can lead to loss of chromosomes, it is difficult to know how to interpret these findings.

Parthenogenesis per se does not increase the susceptibility of oocytes to undergo malsegregation events, as low rates of aneuploidy are observed following hyaluronidase-induced (O'Neill and Kaufman, 1988) and strontium-induced activation (O'Neill et al., 1991). When recently ovulated oocytes are exposed to ethanol, however, high rates of aneuploidy are induced, and the rates of aneuploidy observed appear to be directly related to the duration of exposure to this agent (Kaufman, 1982; O'Neill and Kaufman, 1988). This finding is associated with the presence of lagging chromosomes and anomalies of the meiotic spindle apparatus in the activated oocytes (O'Neill et al., 1989). One of the first responses to a single electrical activation pulse in the mouse is the rotation of the spindle apparatus to a radial position (Gulyas, 1976). While the latter study demonstrated ultrastructural damage to the spindle apparatus and abnormal separation of the chromosomes at anaphase, no follow-up study was unfortunately undertaken to establish the cytogenetic consequences of these observations. It is therefore unclear whether transient or irreparable damage occurs to the spindle apparatus after electrical stimulation. Indirect evidence from the present study suggests that since the latter is not associated with an increase in aneuploidy, the damage produced in the spindle apparatus is probably only transient and readily repaired and probably has no long-term detrimental effect on chromosome segregation.

The spontaneous rate of aneuploidy in one-cell fertilised embryos as observed at metaphase of the first cleavage division in this study was 0.6%, which was not significantly different from the incidence observed with electrically activated oocytes. The low rate of spontaneous aneuploidy in mice observed here is consistent with the findings of others (Donahue, 1972; Rohrborn, 1972; Kaufman, 1973; Dyban and Baranov, 1987), and is

much lower than the estimates of aneuploidy reported to occur spontaneously in man where it has been conservatively estimated that at least 15–20% of all recognised human pregnancies result in spontaneous abortion (Bond and Chandley, 1983). Moreover, 50–60% of the latter are chromosomally abnormal (Carr, 1971; Boué et al., 1975), with trisomics constituting the highest individual component group.

We observed that the activation rate of oocytes exposed to an electric pulse in nonelectrolyte solution was significantly greater than in the various control groups. This was not a postovulatory aging effect, since all groups were matched to account for this factor. It is now widely accepted that it is not the electroactivation *per se* that activates the oocyte, but the influx of  $\text{Ca}^{2+}$  that occurs as a consequence of the electrical stimulus (Onodera and Tsunoda, 1989; Ozil, 1990; Fissore and Robl, 1992; Vitullo and Ozil, 1992; Rickords and White, 1992). Electroactivation is believed to create pores in lipid bilayers and make the membrane more permeable to chemical influx (Zimmermann and Vienken, 1982). In this study, electroactivation occurred in medium that was  $\text{Ca}^{2+}$  free, but if the oocytes remain permeable to extracellular  $\text{Ca}^{2+}$  for some minutes after activation (Rickords and White, 1992), washing in M16 medium in the presence of  $\text{Ca}^{2+}$ , as occurred in this study, would lead to a chemical influx and activation. Presumably, electroactivation in the presence of  $\text{Ca}^{2+}$  might be expected to lead to even higher rates of activation than observed in the present study (Fissore and Robl, 1992; Rickords and White, 1992).

Only a small proportion of recently ovulated oocytes that were not given an electric pulse but were, however, immersed in nonelectrolyte solution for the same period of time as those in the experimental groups successfully activated. Activation in these cases may possibly have resulted from their exposure to  $\text{Ca}^{2+}$ -free medium, which is in any case known to activate oocytes (Surani and Kaufman, 1977; Whittingham and Siracusa, 1978). It was also clear that handling the oocytes and removing their surrounding cumulus cells with hyaluronidase had an activating effect (Kaufman, 1973; Kaufman and Surani, 1974) and the rates observed for oocytes electrically activated and previously exposed to these particular environmental conditions are likely therefore to have been cumulative.

It is now well documented that postovulatory aged oocytes activate more readily than recently ovulated oocytes (Kaufman, 1973, 1983; Webb et al., 1986; Stice and Robl, 1988; O'Neill and Kaufman, 1988; Robl and Stice, 1989; Collas et al., 1989; Ware et al., 1989), but it is unclear whether this is also applicable in the case of human oocytes (Winston et al., 1991). It is important in cloning experiments, however, that recently ovulated oocytes are used, as postovulatory aged oocytes inevitably undergo postovulatory age-related detrimental changes, and are consequently likely to have a reduced long-term viability (Kaufman, 1983).

In the present study, we were able to activate approximately half of the oocytes in the HCG + 15.5 h group,

and it has been found that even very recently ovulated oocytes (i.e., HCG + 12 h) can be activated following repetitive pulse stimulation (Ozil, 1990; Vitullo and Ozil, 1992). The reason why recently ovulated oocytes are apparently difficult to activate experimentally may be related to their response to the electrical pulse, since it has been suggested that intracellular  $\text{Ca}^{2+}$  levels do not change with aging (Fissore and Robl, 1992). It is thought that the level of stimulation employed (i.e., the number and strength of the pulses) and the "maturity" of the oocyte have an effect on the proportion of oocytes that are released from the meiotic "block" (Collas et al., 1989; Kubiak, 1989; Vitullo and Ozil, 1992). Kubiak (1989) reported that oocytes gradually develop the ability for full activation during the period of metaphase II arrest. He found that some oocytes, when subjected to an activating stimulus (provided by sperm or parthenogenetic agent), were able to resume anaphase movement and extrude a second polar body, but were unable to progress into the normal interphase state. These oocytes entered into a so called "metaphase III" phase. Cytogenetic analysis of these oocytes revealed the presence of unichromatid chromosomes. The latter oocytes could be reactivated after a period of a few hours, and this finding would seem to indicate that a certain maturity has to be achieved in order to complete meiosis. Strong parthenogenetic stimuli, such as heat or cold shock, can cause the activation of recently ovulated oocytes, but they also induce considerable damage in the cytoskeletal elements of the oocyte (Kaufman, 1983). In this study, recently ovulated oocytes lysed in greater numbers when subjected to a single electrical pulse than older oocytes, and this finding was also observed by others (see Collas et al., 1989).

Significantly more diploid and immediate cleavage parthenogenones were observed in electrically activated oocytes than in the equivalent control groups. A similar situation is observed following the activation of increasingly postovulatory aged oocytes (Kaufman, 1973, 1983); this aging effect was observed in both experimental and, but to a lesser degree, in the control groups in this study. The modification of the pathways that occurs as a consequence of aging is most likely to result from the loss of the microfilament-rich area that overlies the spindle apparatus and the "drifting" of the latter towards the centre of the egg, hence the retention of both products of the second meiotic division within the egg (Szöllözi, 1971, 1975; Kaufman, 1975; Webb et al., 1986). The formation of immediate cleavage embryos has rarely been recorded in other studies involving the use of electroactivation (Onodera and Tsunoda, 1989; Ozil, 1990; Vitullo and Ozil, 1992). This may be the result of strain differences, but it is more likely that the type of parthenogenone induced is dependent on either the pulse duration given to activate the oocytes or the character (i.e., the frequency and amplitude) of the pulse employed (Ozil, 1990), possibly through interference with the location or functioning of the cytoskeletal elements of the oocyte during the completion of meiosis.



Our findings have clearly demonstrated that the activation of oocytes by a single pulse stimulation does not subject oocytes to a higher than baseline incidence of aneuploidy. The findings of others (see Vitullo and Ozil, 1992) would seem to suggest that repetitive pulse stimulation equally does not interfere with the normal events associated with chromosome segregation that occurs at the time of activation. We believe that improvements in the electroactivation technique and the activation of very recently ovulated oocytes will enable the investigation of mouse parthenogenetic development under more optimal conditions than are used at the present time. Furthermore, these advances in methodology may also facilitate the postimplantation development of parthenogenones beyond the forelimb bud stage, which is the most advanced stage so far achieved by these embryos (Kaufman et al., 1977; Kaufman, 1983).

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# The Cleavage Rate of Digynic Triploid Mouse Embryos During the Preimplantation Period

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**ABSTRACT** Triploidy is a lethal condition in mammals, with most dying at some stage between implantation and term. In humans, however, a very small proportion of triploids are liveborn but display a wide range of congenital abnormalities. In particular, the placentas of human diandric triploid embryos consistently display "partial" hydatidiform molar degeneration, while those of digynic triploids generally do not show these histopathological features. In mice, the postimplantation development of diandric and digynic triploid embryos also differs. While both classes are capable of developing to the forelimb bud stage, no specific degenerative features of their placentas have been reported. Diandric triploid mouse embryos are morphologically normal while digynic triploid mouse embryos consistently display neural tube and occasionally cardiac abnormalities.

Previously it was shown that the preimplantation development of micromanipulated diandric triploid mouse embryos was similar to developmentally matched diploid control embryos. In this study, the preimplantation development of micromanipulated digynic triploid mouse embryos is analysed and compared with that of diandric triploid mouse embryos in order to determine whether there is any difference in cleavage rate between these two classes of triploids.

Standard micromanipulatory procedures were used to insert a female or a male pronucleus into a recipient diploid 1-cell stage embryo. The karyoplast was fused to the cytoplasm of the embryo by electrofusion. These trippronucleate 1-cell stage embryos were then transferred to pseudopregnant recipients and, at specific times after the HCG injection to induce ovulation, the embryos were recovered and total cell counts made. These results were plotted and regression lines drawn.

An additional control group of embryos was subjected to similar micromanipulatory procedures to those used in the experimental study. These embryos had a single pronucleus removed and this was then reinserted into the perivitelline space. Diploidy was immediately restored by electrofusion. These embryos were transferred to recipients and at specific times after the HCG injection the embryos were recovered and total cell counts made. These results were also plotted and regression lines drawn.

The results show that the cell doubling time of the digynic triploid embryos was 14.84 h ( $\pm 1.19$ ). This was not significantly different from that of the diandric triploid embryos (13.55 h  $\pm 0.86$ ;  $P > 0.05$ ) or of the manipulated diploid controls (12.12 h  $\pm 0.79$ ;  $P > 0.05$ ).

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**Key Words:** Digynic triploid, Mouse, Preimplantation embryo, Cleavage rate

## INTRODUCTION

Triploidy is generally lethal during either the embryonic or early fetal period in mammals. In humans, development to term of triploids is very occasionally encountered, however, (e.g., Fryns et al., 1977), but most of these die soon after birth (see Dyban and Baranov, 1987, for review). The majority (85%) of human triploids are thought to be diandric in origin and arise principally through dispermy. The remainder (15%) are digynic, where the extra genetic component is maternal in origin (Jacobs et al., 1982; Surti, 1987). The placentas of the diandric triploid embryos characteristically show "partial" hydatidiform molar degeneration (Carr, 1969; Szulman and Surti, 1978; Szulman, 1987; Kaufman, 1988). This condition is often correlated with a longer than normal gestational period (Jacobs et al., 1982). By contrast, the placentas of human digynic triploid embryos do not show these molar changes, though they may show nonspecific degenerative features (Surti, 1987).

In rodents and rabbits, triploid embryos survive into the early postimplantation period, although no development to term has so far been reported. The development of rat triploid embryos, for example, is reported to be identical to that of diploid embryos up to about 10 days of gestation (Piko and Bomsel-Helmreich, 1960), and rabbit triploid embryos are described as being morphologically normal at fifteen days of gestation and only retarded by about one day compared to that of normal diploid embryos (Bomsel-Helmreich and Thibault 1962; Bomsel-Helmreich, 1971). Spontaneously occurring and experimentally induced triploid mouse embryos are also capable of surviving up to about 10 days of gestation (Fischberg and Beatty, 1951; Vickers, 1969; Baranov, 1976; Kaufman and Speirs, 1987), but their development is generally somewhat retarded.

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The proportionate incidence of the type of triploidy encountered, whether diandric or digynic, is generally very species specific (see Dyban and Baranov, 1987), but in certain strains of mice, no obvious selection appears to exist (Donahue, 1972a,b). Digynic triploidy occurs spontaneously in many mammalian species, and usually results from an error of cytokinesis that may occur during either the first or the second meiotic division of the oocyte. More rarely, it may result from the fertilisation of a "giant" diploid oocyte by a normal haploid sperm (Funaki and Mikamo, 1980; Funaki, 1981).

Digynic triploids are technically easier to produce experimentally than are diandric triploid embryos. Superovulation per se is reported to increase the incidence of digynic triploidy (Takagi, 1970; Takagi and Oshimura, 1973; Takagi and Sasaki, 1976; Maudlin and Fraser, 1977; Speirs and Kaufman, 1988), as well as postovulatory aging of the oocyte (Shaver and Carr, 1967; Vickers, 1969; Yamamoto and Ingalls, 1972), where the drifting of the meiotic spindle apparatus from the periphery towards the centre of the egg can lead to failure of extrusion of the second polar body following fertilisation (Szöllösi, 1971). Heat treatment (Beatty and Fischberg, 1949) and exposure to cytoskeletal inhibitors, such as cytochalasin B or D and colcemid, can also inhibit second polar body extrusion (Bomse-Helmreich, 1965; Niemierko, 1975, 1981; Surani and Barton, 1983; Speirs and Kaufman, 1989a). Similarly, the exposure of recently fertilised embryos to a strong osmotic shock can lead to the incorporation of the second polar body back into the embryo, and in 30–60% of appropriately treated embryos, digynic triploidy resulted (Opas, 1977). The incidence of triploidy is also increased following in vitro fertilisation (IVF) in all species studied, though this is mostly believed to result from polyspermy (Fraser et al. 1976; Wentz et al., 1983; Van Der Ven et al., 1985; Plachot et al., 1985). In one recent report, however, approximately 10% of the triploid embryos obtained following IVF treatment were believed to be digynic in origin (Restagno et al., 1988).

Relatively little is known about the effect that a triploid genome has on development. Digynic triploid mouse embryos, for example, frequently display craniofacial abnormalities which may be associated with axial neural tube defects, and some embryos may additionally have cardiovascular abnormalities. These embryos are smaller than developmentally matched controls but are capable of surviving up to the forelimb bud stage (Kaufman and Speirs, 1987; Kaufman, 1991). In many strains of mice as well as in other mammalian species, a "triploidy syndrome" is encountered where only an empty gestational sac is formed (Wróblewska, 1971). Diandric triploid mouse embryos are also capable of surviving up to the forelimb bud stage and possess about 25 pairs of somites. Although they are smaller than developmentally matched controls, they have a relatively normal morphology and, unlike the situation in man, the morphology of their extraembryonic tissues appears to be normal (Kaufman et al., 1989a; Kaufman et al., 1989b). Androgenetic (i.e., dian-

dric diploid) conceptuses, however, show poor embryonic growth with a maximum of only about 6–8 pairs of somites, but they have extensive proliferation of the trophoblast (Barton et al., 1984). The latter is consistent with the current view that the female and male genomes have different but complementary roles during early mammalian development, where the female genome is believed to regulate embryogenesis while the male genome plays a role in the differentiation of the extraembryonic tissues (McGrath and Solter, 1984; Mann and Lovell-Badge, 1984; Surani et al., 1984, 1986).

The variation in the phenotype achieved between diandric and digynic triploid mouse embryos and their smaller size than that of developmentally matched control diploid embryos may arise, at least in part, from an abnormally slow rate of cellular proliferation during the early postimplantation period, possibly as a consequence of their genetic imbalance. We believe that this is a possible explanation since we have observed that diandric triploid embryos produced by nuclear manipulation divide during the preimplantation period at the same rate as diploid controls and, moreover, that they achieve the blastocyst stage at the same time (Henery and Kaufman, 1992a). While some authorities have reported that digynic triploid mouse embryos develop at the same rate as controls (e.g., Niemierko, 1975; Opas, 1974; Funaki, 1981), others have reported that they have a slower rate of cleavage (Takagi and Sasaki, 1976; Beatty and Fischberg, 1951). To date, however, no detailed analysis has been made that would allow a comparison to be made between the cleavage rate of these two classes of triploids. This represents the basis for the present study.

## MATERIALS AND METHODS

In order to show unequivocally that our procedure for producing digynic triploid embryos was satisfactory, Rb(1.3)1Bnr male mice were used. The genotype of homozygous Rb(1.3)1Bnr males ( $2n = 38$ ) contains two large metacentric chromosomes, being Robertsonian translocations involving chromosomes 1 and 3 (cf. in normal mice  $2n = 40$ ). Consequently, following fertilisation by spermatozoa from these males, the paternally derived haploid genome contains 18 acrocentric and 1 large metacentric "marker" chromosome. Thus, digynic triploid embryos, which have two maternally derived haploid chromosome sets and one normal male chromosome set derived from the homozygous Rb(1.3)1Bnr males, would invariably be expected to contain a total complement of 59 chromosomes, one of which would be the large metacentric "marker" chromosome. Diandric triploid embryos, by contrast, would invariably be expected to contain a total complement of 58 chromosomes, two of which would be the large metacentric "marker" chromosomes.

### The Preimplantation Development of Digynic Triploid Embryos

Eight- to 12-week-old (C57BL  $\times$  CBA)F1 hybrid female mice were injected with 5 IU of pregnant mare

serum gonadotrophin (PMSG), followed 48 h later by 5 IU of human chorionic gonadotrophin (HCG). Shortly after the HCG injection, the females were caged with fertile homozygous Rb(1.3)1Bnr male mice. Early the following morning, the females were checked for the presence of a vaginal plug, and the latter was taken as evidence of mating. The morning of finding a vaginal plug was considered to be the first day of pregnancy.

Early pronucleate-stage fertilised eggs were isolated at about 10 A.M. in the morning on the day of finding a vaginal plug. In these early fertilised eggs, the female pronucleus is always located in close proximity to the second polar body, while the male pronucleus is located elsewhere in the cytoplasm, but usually in the subcortical zone located at the periphery of the egg.

The fertilised eggs were incubated for 45 min in M2 medium (Quinn et al., 1982) supplemented with 1 µg/ml of cytochalasin D and 1 µg/ml of colcemid, prior to microinjection. Female pronuclei were isolated with a small volume of cytoplasm from "donor" eggs and inserted into the perivitelline space of "recipient" 1-cell stage fertilised eggs, using standard micromanipulatory techniques (McGrath and Solter, 1983). Batches of these fertilised eggs with injected donor pronuclei were washed through drops of M16 medium (Whittingham, 1971) and then incubated for 1 hour in M16 at 37°C in an atmosphere of 5% CO<sub>2</sub> in air.

The injected pronuclei were then fused to the fertilised egg cytoplasm by electrofusion. The embryos that were to be exposed to this procedure were transferred into a nonelectrolyte solution consisting of 0.3M mannitol (Kubiak and Tarkowski, 1985); a similar solution was also present in the fusion chamber. The latter consisted of a plastic tissue culture dish that had two platinum wires, each of 250 µm-diameter fixed approximately parallel to each other on the bottom of the fusion dish with a space of about 600 µm between them. The ends of the platinum wires were connected to a digitimer pulse stimulator set at 100 mV with a pulse duration of 50 µs. The manipulated embryos were individually placed between the wires to ensure that the injected pronucleus was orientated approximately parallel to the wires, and the pulse stimulator was triggered. The embryos were removed immediately and washed through 4 drops of M16 and then returned to the incubator.

Fusion usually occurred within an hour. These digynic triploid trippronucleate embryos were then transferred unilaterally to the oviducts of recipients on the first day of pseudopregnancy (i.e., on the first day of finding a vaginal plug after spontaneously cycling females had been mated to proven sterile vasectomised males). The recipients were anaesthetised with tribromoethanol (Avertin: Winthrop; dose 0.02 ml/g body weight of a freshly prepared 1.2% solution of Avertin dissolved in 0.9% saline).

At various times between 65–96 h after the HCG injection, the recipient females were killed by cervical dislocation. The oviducts or uteri on the operated side were removed and flushed through with phosphate buffered saline (PBS) containing 4% bovine serum al-

bumin (BSA) in order to recover the transferred embryos. The embryos were then incubated for 3–4 h in the presence of colcemid to facilitate the determination of their chromosome constitution.

In all instances, note was taken of the gross morphological appearance of individual embryos, whether they were at cleavage stages, precompacted or compacted morulae or blastocysts, and from which location within the reproductive tract they were recovered. Preparations were made of these embryos using the air-drying technique described by Tarkowski (1966) and the spreads were then stained with 10% Giemsa to permit accurate cell counts.

### Preimplantation Development of Diandric Triploids

Another group of superovulated F1 hybrid females was mated to fertile homozygous Rb(1.3)1Bnr males and the male pronuclei inserted into the perivitelline space of recipient eggs as described above. After electrofusion, these diandric triploid trippronucleate embryos were also transferred unilaterally to the oviducts of recipients. At various times between 49–100 h after the HCG injection, these recipients were killed and the embryos recovered as described above. Preparations were also made of these embryos to establish cell numbers.

### Control Series

In the control series, F1 hybrid females were mated to homozygous Rb(1.3)1Bnr males. The male pronucleus was removed (although, at least theoretically, it would have been equally as valid to remove the female pronucleus) via an injection pipette and then reinserted back into the perivitelline space where, after electrofusion, it subsequently fused to the cytoplasm to restore the diploid status of these embryos. They were then transferred to pseudopregnant recipients and isolated at specific time points after the HCG injection when their total cell number was established as described previously.

## RESULTS

### Cleavage Rates

After the embryos from each group had been isolated from the recipients at specific times after the original HCG injection, their total cell count was established. The values obtained were converted into natural logarithms and plotted against time after HCG. Regression lines were drawn and this allowed a cell doubling time for each group to be established. Since the detailed findings from series II and III have previously been published (Henery and Kaufman, 1992a), only the information relevant to this study is presented here.

### The Preimplantation Development of Digynic and Diandric Triploid and Control Diploid Embryos

The digynic triploid embryos had a cell doubling time of 14.84 h ( $\pm 1.19$ ) between 48–96 h after the original HCG injection. Diandric triploid embryos had a cell doubling time of 13.55 h ( $\pm 0.86$ ) between 49–100 h

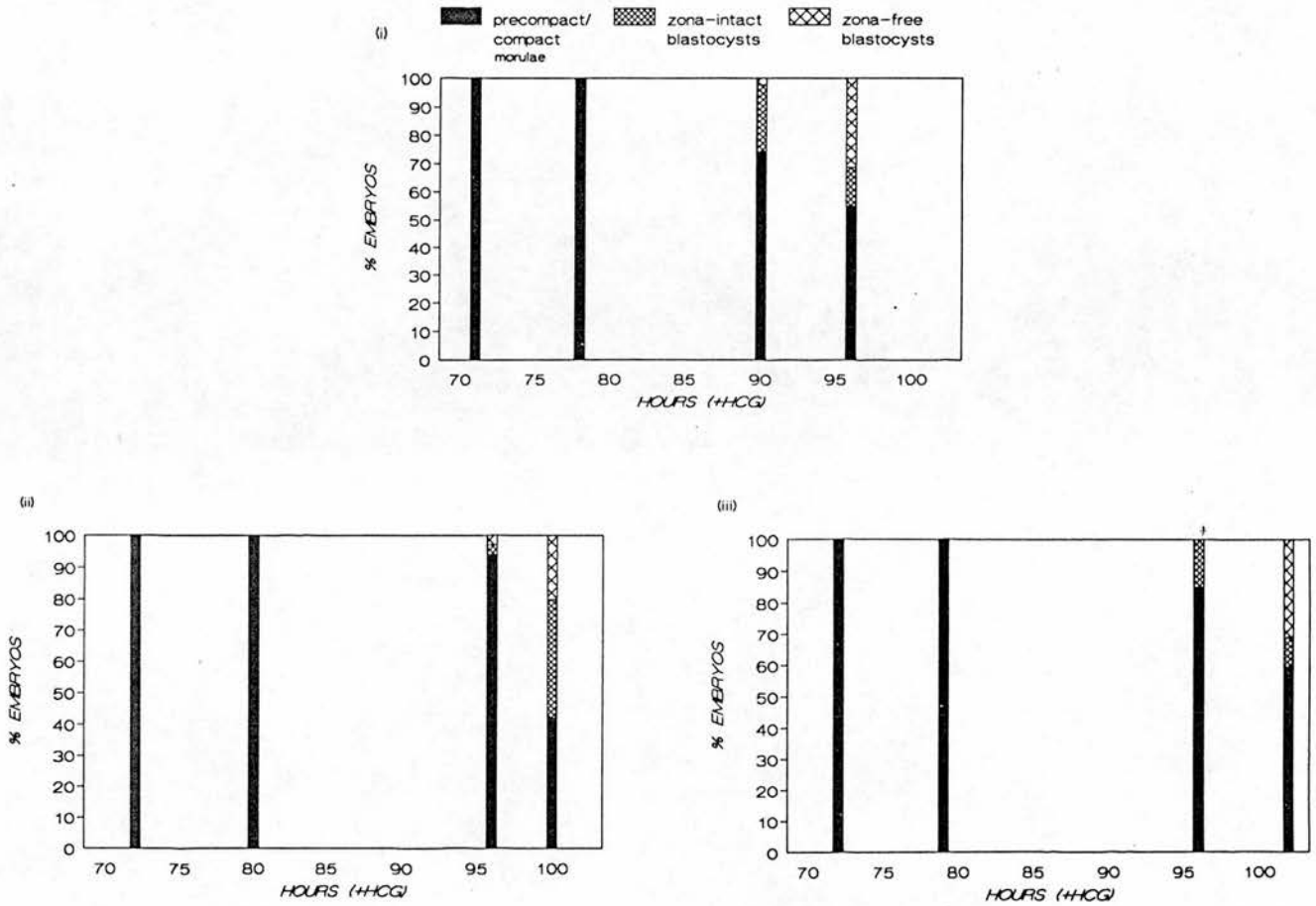


Fig. 2. Morphological appearance of embryos isolated at various times during the preimplantation period, plotted against time after HCG injection to induce ovulation in groups (i), (ii), and (iii) as in Figure 1.

(6%) than the digynic triploids by 96 h after HCG, but by 100 h, about 58% of the embryos recovered were blastocysts and about 20% of these were found to be zona-free.

In the micromanipulated control group recovered and analysed at 96 h, 15% of the embryos were blastocysts, and by 102 h, this had increased to about 41% with a large proportion of them being zona-free (31%). The findings from this component of the study are presented diagrammatically in Figure 2. It is clear from this component of the study that advanced preimplantation stages of development can be achieved by embryos that contain relatively low numbers of component cells, as has been consistently observed by others (see, e.g., Kelly, 1975).

## DISCUSSION

Digynic and diandric triploid mouse embryos develop up to the early postimplantation period, but fail to progress beyond the limb bud stage (Kaufman and Speirs, 1987; Kaufman et al., 1989a,b). In the present study we have demonstrated that digynic triploid embryos produced by nuclear transfer cleave at the same

rate as similarly manipulated diploid controls. This complements work undertaken in a previous study in which we demonstrated that diandric triploid embryos, produced by nuclear manipulation, also cleaved at the same rate as appropriately prepared controls (Henery and Kaufman, 1992a). In these experiments, the technique employed to produce digynic triploid embryos, may have resulted in a greater degree of genetic heterozygosity than would have occurred following suppression of the second polar body, but we believe that this factor has probably a negligible effect on cleavage rate during the preimplantation period.

Despite the fact that, during the preimplantation period, digynic and diandric triploid mouse embryos are morphologically remarkably similar, the postimplantation morphological appearance of these two classes of embryos are quite different. The digynic triploid embryos characteristically display neural tube and often cardiac abnormalities, while the diandric triploids, though smaller than developmentally matched controls, are nevertheless usually morphologically normal (Kaufman et al., 1989a; Kaufman, 1991). These differences probably arise as a result of genomic imprinting



TABLE 1. Preimplantation Development of Digynic Triploid Mouse Embryos

| Group            | Time of isolation (hours post-HCG) | No. embryos transferred | No. embryos recovered (healthy) | No. analysed to establish mean cell number | Mean cell number ( $\pm$ SEM) |
|------------------|------------------------------------|-------------------------|---------------------------------|--|-------------------------------|
| Digynic triploid | 48                                 | 12                      | 10                              | 10   | 2.00 ( $\pm$ 0)               |
|                  | 65                                 | 59                      | 45                              | 41   | 6.00 ( $\pm$ 0.31)            |
|                  | 71                                 | 55                      | 47                              | 37   | 6.05 ( $\pm$ 0.31)            |
|                  | 78                                 | 51                      | 37                              | 36   | 9.47 ( $\pm$ 0.58)            |
|                  | 90                                 | 86                      | 46                              | 39   | 15.28 ( $\pm$ 1.06)           |
|                  | 96                                 | 77                      | 50                              | 40   | 23.20 ( $\pm$ 1.80)           |

Comparable results from the analysis of the diandric triploid and manipulated diploid control embryos are published in Henery and Kaufman (1992a), and the findings are summarised under Results (see also Fig. 1).

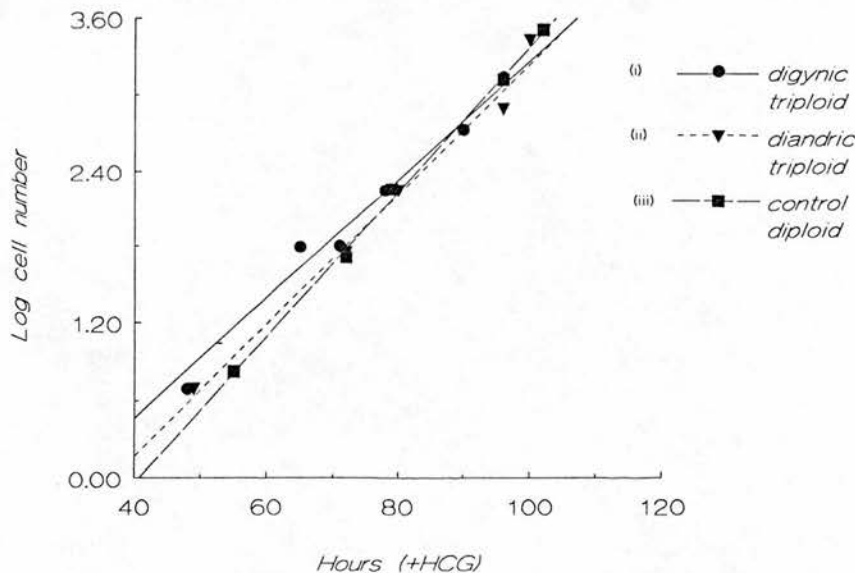


Fig. 1. Log total cell number of embryos isolated at various times during the preimplantation period, plotted against time after HCG injection to induce ovulation in (i) digynic triploid embryos (ii) diandric triploid embryos (iii) micromanipulated diploid control embryos.

after HCG, while the cell doubling time for the micromanipulated control diploids was 12.12 h ( $\pm$ 0.79) between 55–102 h after HCG.

The regression lines plotted for these embryos showed that digynic triploid mouse embryos produced by the technique of nuclear manipulation divide at the same rate as diandric triploid embryos ( $P > 0.05$ ) and manipulated diploid controls ( $P > 0.05$ ) produced under the same experimental conditions. Previously we demonstrated that the stresses of nuclear manipulation on an embryo do not appear to detrimentally affect its cleavage rate during the preimplantation period. Similarly, exposure to cytoskeletal inhibitors during the micromanipulatory procedure appears to have little or no detrimental effect on their cleavage rate (Henery and Kaufman, 1992a). The findings from this study are summarised in Table 1, and the regression lines are presented in Figure 1.

#### Location and Morphology of the Preimplantation Embryos

By definition, the precompacted and compacted morulae had yet to form a blastocoelic cavity at the

time of their isolation. All blastocysts, however, had a visible blastocoelic cavity, and this group included both early cavitating and fully expanded blastocysts. Zona-free blastocysts were those embryos that had hatched from within their zona pellucida.

At 71 h after the HCG injection, the digynic triploids were found exclusively in the oviduct and were all either precompacted or compacted morulae. This was also the case at 78 h. By 90 h, however, some had progressed to the blastocyst stage. A small proportion of the latter were zona-free, and were located in both the oviduct and uterus. The mean cell number at 90 h was 15.28 ( $\pm$ 1.06). By 96 h, about 45% of the embryos recovered were at the blastocyst stage and these were mostly located in the uterus. A proportion of these blastocysts were zona-free (31.4%). Because preliminary experiments had indicated that the efficiency of recovery of triploid embryos at points after 96 h after the HCG injection was relatively poor, such groups were not studied.

The diandric triploid embryos in general followed a similar progress to that of the digynic triploid embryos. A smaller proportion had reached the blastocyst stage

where the parental origin of genes determines their expression (Cattanach, 1985; Surani et al., 1986; Solter, 1988). Duplication of one set of parental chromosomes (associated with the absence of the complementary chromosome, but of the other parental origin) may cause different phenotypic traits within the embryo through changes in the balance of gene dosage required for normal development (Epstein, 1986; Barton et al., 1991). In the human, a wide range of congenital abnormalities are commonly encountered in triploid embryos (Harris et al., 1981), although no triploidy syndrome complex has yet been reported. The placentas of diandric triploid embryos invariably show "partial" or "incomplete" hydatidiform molar degeneration, though the placentas of the digynic triploid class display no consistent abnormalities (Szulman and Surti, 1978; Surti, 1987).

It has been postulated that the sex-chromosome constitution of triploid embryos may have an influence on the severity of the pathological changes found (Edwards et al., 1967). However, such a simple relationship was not observed when the sex-chromosome constitution of digynic triploid embryos from LT/Sv strain mice was analysed at ten days of gestation. In this instance, the sex ratio of XXX:XXY (the only possible sex-chromosome constitutions that are encountered in these embryos) observed was the predicted one, namely 1:1 (Speirs and Kaufman, 1989b). By contrast, in diandric triploids, the sex-chromosome constitution of the embryo does appear to influence its developmental potential. While mouse embryos with either an XXX or an XXY sex-chromosome constitution are capable of achieving the forelimb bud stage, the development of those with an XYY constitution is restricted, in that they rarely develop beyond the early somite stage (Kaufman et al., 1989b). This is the likely explanation for the clinical observation that the observed ratio of XXX:XXY:XYY is close to 11:19:1 (Niebuhr, 1974; Hassold et al., 1980; Uchida and Freeman, 1985) rather than 1:2:1 had the development potential of these three classes of diandric triploid embryo been unaffected by their sex-chromosome constitution.

The development potential of triploid embryos may also be detrimentally affected as a consequence of geometric problems, and abnormal cell-cell spatial interactions. The nucleated primitive red blood cells of digynic and diandric triploid mouse embryos, for example, have a cell and nuclear volume which is significantly larger than comparable cells in developmentally matched diploid control embryos (Henery and Kaufman, unpublished). A similar relationship has also been described for non-nucleated triploid red blood cells in the rabbit (Bomsel-Helmreich, 1971). Since they invariably have a smaller body size than developmentally matched controls, triploid embryos, therefore, must contain less cells than diploid embryos. This pattern is similar to that observed in amphibia and fish, which can nevertheless attain normal development and may even be fertile and capable of reproducing (Fankhauser, 1941; Small and Benfrey, 1987). In mam-

mals, however, such a reduction in their total cell number may be one of many reasons why these embryos die.

It has recently been hypothesised that in mouse tetraploids, an increase in cell size together with a concomitant reduction in cell number may explain the severe forebrain abnormalities encountered in this group (Snow, 1975; Henery and Kaufman, 1992b; Henery et al., 1992). The reduction in total cell number must commence at or shortly after implantation or during the early postimplantation period, since triploid and diploid embryos appear to have the same total number of cells at comparable stages during the preimplantation period. Studies on early postimplantation aggregation chimeric embryos have shown that this is also a time when embryonic size regulation is thought to occur (Tarkowski, 1963; Buehr and McLaren, 1974).

It has been suggested that a change in the nucleocytoplasmic ratio of developing embryos may also have a deleterious effect on their development (Barton and Surani, 1983). It is clear that haploid embryos do not develop as well as diploid embryos during the preimplantation period, and blastocyst stages of development are less commonly achieved (Modliński, 1975; 1980; Kaufman and Sachs, 1976; Kaufman, 1981, 1983; Borsuk, 1982; Henery and Kaufman, 1992c). McGrath and Solter (1986) demonstrated that the removal of a relatively small volume of the cytoplasm of 1-cell haploid embryos significantly increased their development potential, as well as increasing the proportion of embryos that successfully achieved the blastocyst stage. In a complementary series of experiments, Evsikov et al. (1990) reported an estimated 30% reduction in cleavage rate when approximately one third of the cytoplasm was removed from 1-cell fertilised embryos.

Triploid embryos inevitably have an altered nucleocytoplasmic ratio compared to diploid controls, but this does not appear to detrimentally affect the preimplantation development of these embryos (Henery and Kaufman, 1992a). It appears therefore that the decreased nucleocytoplasmic ratio observed in haploid embryos is detrimental to their development, while an increase in this ratio (closer to that of the controls, which is presumably the optimal ratio) is compatible with their further development. Embryos which contain multiples of the haploid genome (as occurs in the case of the triploids) can clearly develop normally during the preimplantation period, and it is only later in development that problems arise. The latter may be due either to lack of a complementary parental genome, or from alterations in gene dosage or from geometric problems which might eventually lead to the premature death of these embryos (Epstein, 1986).

It is relevant to note here that homozygous tetraploid embryos produced by electrofusion of blastomeres at the 2-cell stage (Kaufman and Webb, 1990) have a "normal" nucleocytoplasmic ratio. Furthermore, their cleavage rate during the preimplantation period is identical to that of normal diploid embryos, despite the fact they only possess about half the total number of cells normally present at comparable stages of develop-

ment (Henery and Kaufman, 1991). More interestingly, at least a proportion of tetraploid mouse embryos are capable of significantly more advanced stages of development than haploid or triploid embryos, the most advanced tetraploid embryos analysed to date developing to a stage equivalent to about 14.5 days p.c. of normal diploid development (Kaufman, 1992).

Our findings clearly demonstrate that polyploid embryos cleave at the same rate as normal diploid embryos (Henery and Kaufman, 1991, 1992a,c) at least during the preimplantation period. Previous investigators had indicated that the mean number of nuclei present in polyploid embryos of the same developmental age was approximately in inverse proportion to the number of chromosome sets present (Beatty and Fischberg, 1951). The findings were, however inconsistent, with some authorities reporting that triploid embryos divided more slowly than diploid embryos (Baranov, 1976; Takagi and Sasaki, 1976), while others observed that there was no difference (Edwards, 1958; Opas, 1974; Niemerko, 1975; Funaki, 1981). Human triploid cell lines maintained in tissue culture are also believed to have a normal cell division rate (Kuliev et al., 1975).

In the light of our findings reported here, we believe that it would be of considerable interest therefore to investigate further the development (and cellular morphology) of the trophectoderm and inner cell mass components of triploid embryos, as alterations to the normal ratio of cells expected at this early stage of embryonic development may account for their morphological features observed during the early postimplantation period.

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